Mechanisms of Transcriptional Modulation of Gene Expression by Oestrogen Receptors

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Declaration

The research described in this thesis is the sole work of the author and is not being submitted in support of another degree or qualification at this university or any other university or institute of learning.

Acknowledgements

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Just a little note to Michelle; I hope I haven't put you off doing a PhD...it is worth all the hard work and perseverance! So good luck to Sharon, Michelle and Nina with your PhDs!

I also would like to thank my family for their continual support over the last three years.

Abbreviations

AR	Androgen receptor
ARKO	Androgen receptor knock-out
bp	Base pairs
CsCl	Caesium chloride
DNA	Deoxyribose nucleic acid
DPN	2,3-bis(4-Hydroxyphenyl)-propionitrile
DsRed	Clontech's fluorescent red vector
EDTA	Ethylene diamineteta-acetic acid
EGF	Epidermal growth factor
EGFP	Clontech's fluorescent green vector
EGFr	Epidermal growth factor receptor
ER	Oestrogen receptor
ERα	Oestrogen receptor alpha
ERβ1	Oestrogen receptor beta 1 Wild type
ERβ2	Oestrogen receptor beta 2 Variant (also known as ERβcx)
ERE	Oestrogen receptor response element
E_2	17β-Oestradiol
ERKO	Oestrogen receptor knock-out
FP	Fluorescent Protein
g	Gram
GFP	Green fluorescent protein
HCl	Hydrogen chloride
H_2O_2	Hydrogen peroxide
HRP	Horse-radish peroxidase
IGF	Insulin-like growth factor
IGFr	Insulin-like growth factor receptor
kDa	Kilo Dalton
KOAc	Potassium acetate
l	Litre
M	Molar
ml	millilitre
Mc/Mac	Macaque

Mm/Marm	Marmoset
mRNA	messenger RNA
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NaOAc	Sodium acetate
NRS	Normal Rabbit Serum
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPT	4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TE	Tris EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
v/v	Volume to volume ratio
w/v	Weight to volume ratio

Abstract

Oestrogens are essential regulators of both male and female fertility as well as playing an important role in other tissues including the cardiovascular system and bone. Oestrogen action is mediated via high affinity nuclear receptors expressed in target tissues. The first oestrogen receptor (ER) was cloned in 1986 however, ten years later a second ER was identified. The two ERs are known as ERα and ERβ and are encoded by genes on different chromosomes. Splice variants of ERβ have been described in human; one of these variants (known as ERβ2 or ERβcx) contains an alternatively spliced exon at the 3' end. The resulting ERβ2 variant protein has a truncated C-terminal when compared to the ERβ wild type (WT) protein, resulting in the absence of a functional AF-2 region and part of the ligand binding domain, leaving intact the AF-1 domain which may be a target for phosphorylation.

The aims of this project were a) to determine whether ERβ WT and ERβ variant (ERβ2) forms exist in primates as well as in humans and to determine the ER localisation in reproductive tissues, b) to examine the localisation of FP-tagged ER protein constructs (ERα, ERβ WT and ERβ2) within cells using a confocal microscope in the presence or absence of different oestrogenic ligands, c) to examine the functional dose response of ER proteins to different ligands using transient transfections in the presence of ERE-luciferase reporter constructs, d) to investigate an alternative activation mechanism for ERβ2 via a growth factor signalling pathway.

RT-PCR results demonstrated that mRNA for ERβ WT and the ERβ2 variant were differentially expressed in tissues from the human, Old World (stump-tailed macaque) and New World (common marmoset) primates. Immunohistochemical analysis resulted in detection of ERβ WT protein in both human and primate tissues but ERβ2 variant protein immunostaining was only positive in the human tissues. Sequence analysis revealed that the peptide used to raise the anti-hERβ2 antibody was not conserved in either primate. In human tissues, ERβ WT and ERβ2 had differential but overlapping patterns of expression so that formation of homo- or heterodimers might occur. In the absence of ligand the FP-tagged ERs were diffusely distributed within the nucleus of transfected cells. Addition of oestrogenic

ligands induced rapid redistribution of both the ERα and ERβ WT receptors so that they adopted a 'focal' nuclear pattern. The ERβ2 variant remained in a diffuse distribution throughout the nucleus even in the presence of ligand. Transient transfection studies revealed differences in the extent to which ERα, ERβ WT and ERβ2 variant can stimulate gene transcription of ERE-luciferase reporter constructs following addition of oestrogenic ligands. ERα and ERβ WT were both able to stimulate reporter gene expression in a dose-dependent manner, whereas the ERβ2 variant was not able to activate the ERE-luciferase reporter. Co-transfections of ERα or ERβ WT with ERβ2 suggested a possible dominant negative effect of ERβ2 on ERα but this was abolished when the co-activator SRC-1 was present. The ERβ2 variant did not respond to ligand by showing a change in subnuclear localisation nor did it activate the ERE-luciferase consistent with the absence of a functional AF-2 region but it did appear to be activated by epidermal growth factor acting via the MAP kinase phosphorylation pathway.

In conclusion, ERβ WT and ERβ2 variant proteins are expressed in reproductive tissues in human and primate. The ERβ2 variant cannot be activated by binding oestrogens but appears to be a target for phosphorylation via growth factor stimulated pathways. The ability of this receptor to form heterodimers with ERα or with ERβ WT thereby altering target cell responsiveness to oestrogens may have an impact on cell function. Further studies are required to elucidate the relative contributions of ERα, ERβ WT and ERβ variants in the response of tissues to oestrogens and growth factors.

Contents

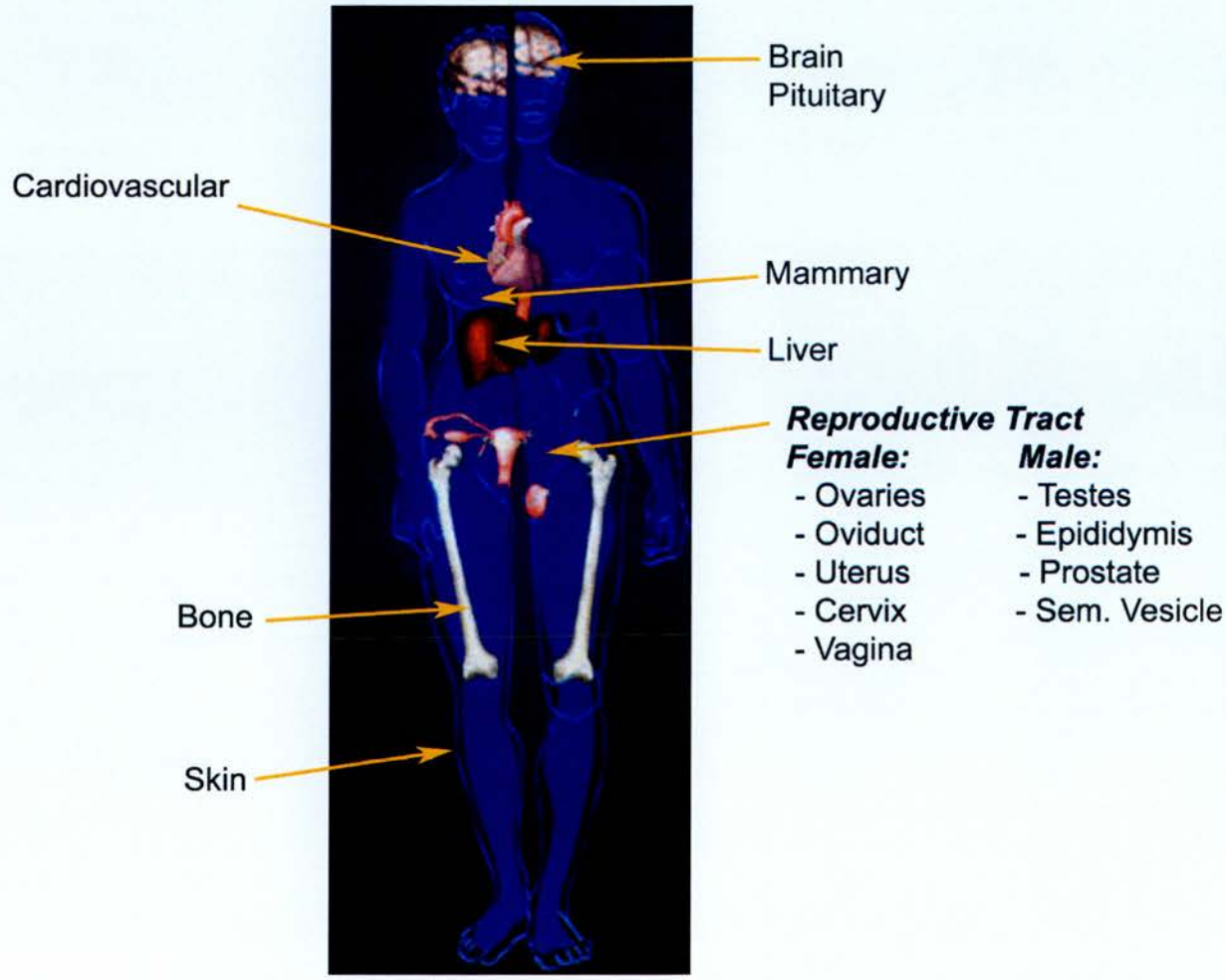
Chapter 1

Literature Review

1.1 Oestrogens and Human Health

Oestrogens influence cell function in a wide range of tissues including bone (Turner *et al.*, 1994), the cardiovascular system (Farhat *et al.*, 1996), brain and central nervous system (McCarthy, 1996) and have an important role in both male and female reproductive function (Clarke *et al.*, 1992; Manolagas and Kousteni, 2001) (see *Figure 1.1*).

Figure 1.1. Figure to demonstrate oestrogen target tissues within the male and female. Adapted from Couse (unpublished).



1.1.1 Oestrogen Action in the Female

A key focus of oestrogen action in the female is within the reproductive system. The menstrual cycle in the female has two distinct phases; 1) the oestrogenic (follicular) phase in which the ovary prepares the female for receipt of spermatozoa and fertilization of the oocyte, 2) the progestagenic (luteal) phase when the ovary prepares the female to receive and nuture the conceptus should fertilisation be successful. See *Figure 1.2.* for details of the hormone levels throughout the cycle.

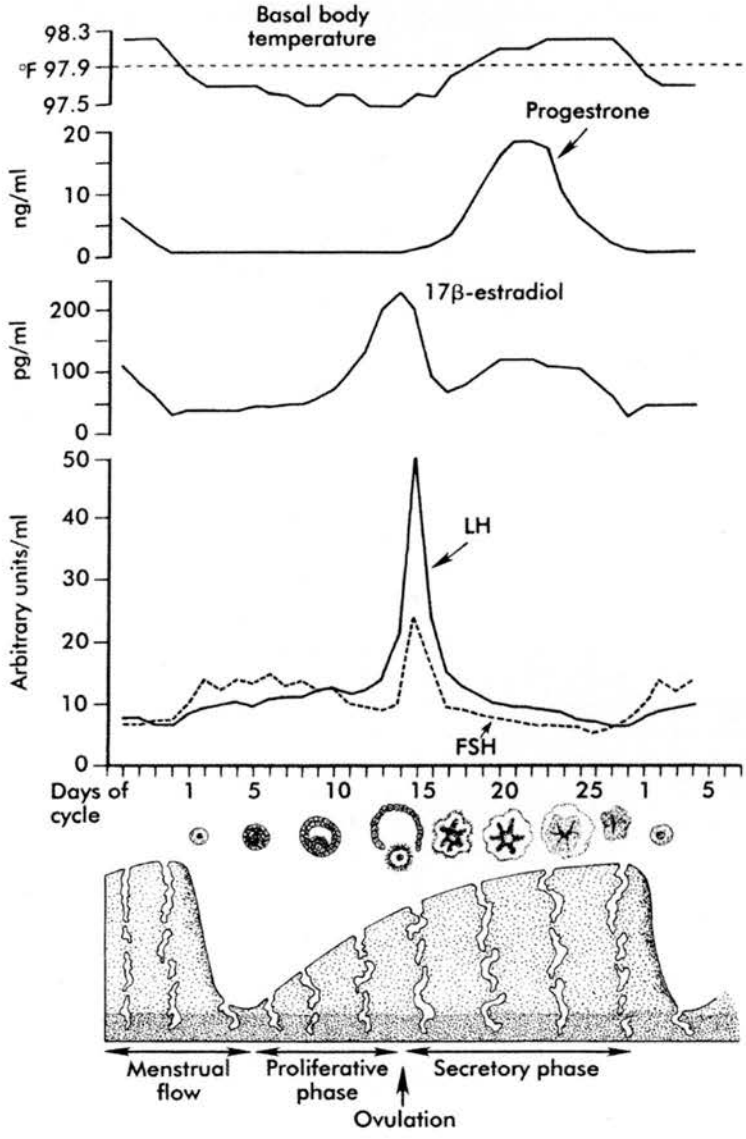


Figure 1.2. Figure to show the levels of oestradiol, progesterone, LH and FSH throughout the human reproductive cycle. Taken from Carlson (1994).

1.1.2 Oestrogen Action in the Male

Oestrogens have an impact on the reproductive and non-reproductive tissues in males (see *Figure 1.1*) and are believed to play a significant role in maintaining fertility (reviewed by O'Donnell *et al.*, 2001). The primary function of the testis is to synthesise and secrete steroid hormones, notably testosterone and to release mature spermatozoa (*Figure 1.3*).

Spermatogenesis involves four basic processes: spermatogonial development (stem cell and subsequent cell mitotic divisions), meiosis (DNA synthesis and two meiotic divisions to yield haploid spermatids), spermiogenesis (spermatid development involving differentiation of head and tail structures) and spermiation (the process of release of mature sperm into the tubule lumen). The organisation of spermatogenesis is essentially the same in all mammals and has been described at the morphological level (Sharpe, 1994) and recently reviewed by McLachlan *et al.*, (2002). In rodents and some primate species (e.g., *Macaca fascicularis*), germ cell development occurs in orderly and recognizable cell associations (or stages) along the seminiferous tubule, such that a single stage can be seen within a tubule cross section (Russell *et al.*, 1990). However, in humans and some other primates (e.g. *Callthrix jacchus*, the common marmoset), the stages are arranged in an intertwining helical pattern such that a single tubule cross section may have up to six identified stages represented (Millar *et al.*, 2000; Schulze and Rehder, 1984). Such an arrangement makes the systematic stage-based counting of germ cell populations, as well as the stage-specific detection of proteins or mRNA species of interest, difficult although possible (McLachlan *et al.*, 2002) and means the marmoset is considered a useful model for human spermatogenesis.

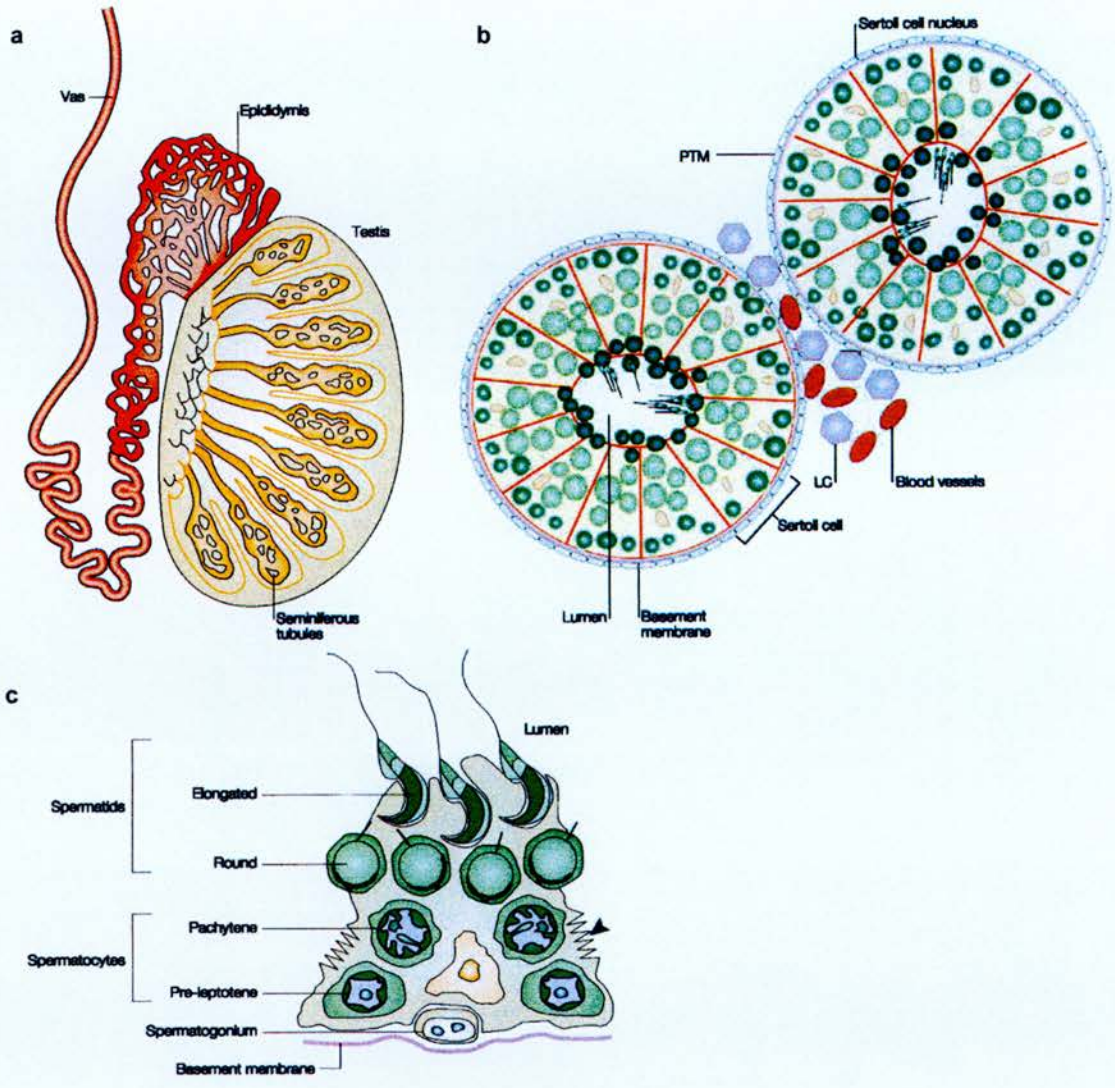


Figure 1.3. Schematic picture of the testes and spermatogenesis (Cooke and Saunders, 2002). A cross section through the testis (a) showing the localisation of the seminiferous tubules, the vas deferens and the epididymis. A diagrammatical cross-section through a testicular tubule (b) showing the germ cells (green) at different stages of maturation within the Sertoli cell (outlined in red). Leydig cells (LC) present in the interstitium, are the sites of testosterone synthesis. Maturing sperm are shown in the lumen of the tubules. PTM; Peritubular myoid cells. A single Sertoli cell (c) with its associated germ cells. Tight junctions between Sertoli cells (arrowhead) define two compartments; the stem cells and the pre-meiotic cells (spermatogonia) are found on one side of the junction whereas meiotic (spermatocytes) and the post-meiotic (round and elongated spermatids) are found organised in order of maturation towards the lumen (cytoplasm is shown in dull green). DNA is shown in pink, Sertoli cell nucleus is shown in orange.

1.2 Steroidgenesis

Oestrogen biosynthesis is dependent on the expression of the cytochrome p450 aromatase enzyme. In females high levels are found in human placenta and the granulosa cells of the mature ovarian follicle (Simpson and Davis, 2001; Turner *et al.*, 2002). This is consistent with the finding that in non-pregnant pre-menopausal females the ovaries are the primary source of oestradiol (Simpson, 2000).

In males, oestrogens are synthesised from testosterone or oestrone via the aromatase or 17β-hydroxysteroid dehydrogenase (17β-HSD) enzymes in the brain and the testis (de Ronde *et al.*, 2003). The total oestradiol production rate in the human male has been estimated to be 35-45μg per day, of which approximately 15-20% is directly produced within the testis (Baird *et al.*, 1969). Therefore oestrogen levels within the reproductive tract of the male are higher than the rest of the circulation (Hess, 2000). The site of oestrogen synthesis in the male was initially thought to be the Leydig cells (Payne, 1990). However more recent data has demonstrated aromatase was also associated with the cytoplasm surrounding elongate spermatids in human, marmoset and rat (Turner *et al.*, 2002) and in rodents, aromatase activity has been found in mature germ cells and spermatozoa (Hess *et al.*, 1995; Nitta *et al.*, 1993).

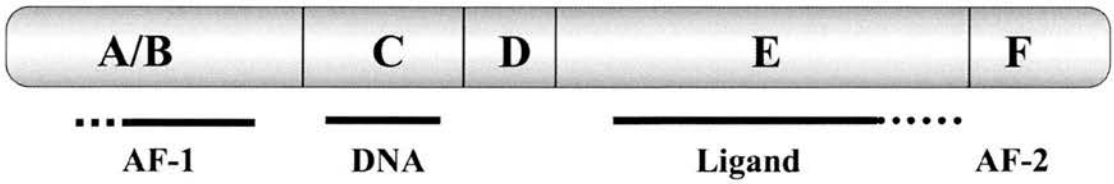
1.3 Nuclear Receptor Gene Superfamily

Nuclear hormone receptors are a class of transcription factors whose activity depends on the binding of a ligand (Parker, 1990). The oestrogen receptor (ER) belongs to the nuclear receptor superfamily, of which there are 83 subgroups identified (NUREBASE; www.ens-lyon.fr/LBMC/laudet/nurebase). These include the receptors for testosterone, progesterone, corticoids, thyroid hormone, vitamins A and D, and a large group of proteins referred to simply as "orphan" receptors for which the specific ligands are still to be determined (Enmark and Gustafsson, 1999). The mechanism of action of these steroid hormones is that upon ligand binding the nuclear receptor undergoes a conformational change which promotes dimerisation, recruitment of co-activator proteins and high affinity binding to specific palindromic DNA response elements, for example the oestrogen response element (ERE) located within the regulatory region of target genes (Beato and Klug, 2000; Mangelsdorf *et al.*, 1995).

1.3.1 Introduction to Steroid Receptor – Group III

Members of the steroid hormone receptor (SHR) family share significant sequence homology with each other and have been classified as subgroup A of the nuclear receptor subfamily 3 (NR3) (reviewed in Beato and Klug, 2000). All the members of the SHR share a common arrangement of five structure-function domains denoted A-F (as pictured in *Figure 1.4*) (Beato *et al.*, 1995; Beato and Klug, 2000; Mangelsdorf *et al.*, 1995), the roles of which have been defined by techniques such as domain switching and site directed mutagenesis (reviewed in Carson-Jurnica *et al.*, 1990). Some domains have high homology between the receptors (e.g. the DNA binding domain), however the A/B and F functional domains are poorly conserved between the family members (Scobie *et al.*, 2002) (see *Figure 1.4*).

Figure 1.4. A diagram to illustrate the nuclear receptor domains. Adapted from Nilsson *et al.*, (2001).



1.3.2 Oestrogen Receptor Genes

The oestrogen receptor family of steroid hormone receptors is multifaceted (Hall, 2001) and more complex than initially thought. The "classical" oestrogen receptor (ERα) was discovered by Elwood Jensen in 1958 and cloned in 1986 from a human breast cancer cell line (Green *et al.*, 1986). For many years it was thought that this was the only ER, until an ERα knockout (ERKO) mouse model was produced and exhibited specific oestrogen binding in some tissues (Lubahn *et al.*, 1993) suggesting that a second oestrogen receptor must exist. In 1996 a second ER (ERβ) was cloned from rat prostate cDNA (Kuiper *et al.*, 1996).

Both oestrogen receptors (ERα and ERβ) are encoded by eight exons, however they are the products of two different genes located on different chromosomes (Enmark *et al.*, 1997). Human ERβ has been mapped to band q22-24 of chromosome 14 (Enmark *et al.*, 1997; Lakaye *et al.*, 1998) whereas human ERα

has been mapped to the long arm of chromosome 6 (Enmark and Gustafsson, 1999).

The ERα and ERβ isoforms have distinct roles in oestrogen action, independent of each other (reviewed in Couse and Korach, 1999) as well as being able to directly interact due to heterodimerisation which may occur when the receptor proteins are expressed together in the same cell (Cowley *et al.*, 1997; Enmark *et al.*, 1997).

1.3.3 Domains

ERα and ERβ have six domains named A-F from the N- to C-terminus, encoded by 8-9 exons (Kuiper *et al.*, 1998) (See *Figure 1.4*). The three major functional domains of the ER are (i) a variable N-terminus (domains A and B) which contains the activation function-1 (AF-1) (Enmark and Gustafsson, 1999; McInerney and Katzenellenbogen, 1996), (ii) a highly conserved DBD, also referred to as the C domain (Enmark *et al.*, 1997) and (iii) a less conserved LBD (E domain) that contains the activation function-2 (AF-2) (Klinge, 2001).

The variable A/B domain modulates transcription by directly interacting with components of the core transcriptional machinery or with co-activators that mediate signalling to downstream proteins in a gene- and cell- specific manner through the AF-1 (Enmark and Gustafsson, 1999).

The DNA-binding domain (C) is the most conserved region between ERα and ERβ, sharing 97% homology (Enmark *et al.*, 1997) and is comprised of two functionally distinct zinc fingers through which the ER interacts directly with the DNA helix. The positions of the cysteine residues within this domain, that co-ordinate the two zinc fingers are very highly conserved. The sequence EGCKAF (hERβ amino acids 122-127) at the base of the first zinc finger is essential for specific binding to oestrogen responsive elements on target genes. The region of the second zinc finger contributes to the receptor dimerisation (CPATNQC; hERβ 143-150). Both zinc fingers are identical in both ERα and ERβ (reviewed in Pettersson and Gustafsson, 2001). As human ERα and ERβ have a conserved DBD it has been proposed that both ERα and ERβ are able to bind to identical oestrogen response elements (EREs) on the DNA (Ogawa *et al.*, 1998a).

The hinge (D) domain contributes flexibility to the DNA and the ligand binding domain and has been shown to influence the DNA binding properties of individual

receptors (Enmark and Gustafsson, 1999). It has also been proposed that the D domain serves as an anchor for certain co-repressor proteins (Enmark and Gustafsson, 1999).

Domain E is located within the C-terminal and contains regions of homology (53%) between ERα and ERβ. It is this region which determines the ligand specificity of the receptor proteins. The LBD also harbours the activation function-2 (AF-2) which is a complex and conserved region whose structure and function are determined by the binding of ligands (Feng *et al.*, 1998; Henttu *et al.*, 1997; Klinge, 2001; Shiau *et al.*, 1998). The C-terminal F domain has been shown to contribute to the transactivational capacity of the receptor via the AF-2 region (Enmark and Gustafsson, 1999).

1.4 The Oestrogen Receptor alpha (ERα)

The ERα protein is approximately 67kDa (Kuiper *et al.*, 1997). The ERα gene (located on chromosome 6) is very large with the translated exons spanning more than 140kb (Enmark *et al.*, 1997; Ponglikitmongkol *et al.*, 1988). The human ERα gene has been shown to have at least three separate promoters (Enmark *et al.*, 1997).

1.4.1 ERα Variants

There are numerous variants of ERα, either with point mutations at a single amino acid or with insertions, or more commonly deletions, which have been identified as mRNAs (Fuqua *et al.*, 1993; Taylor *et al.*, 1998; Zhang *et al.*, 1996).

Examples of ERα isoforms include one with exon 3 deleted (ERαΔ3) and which is therefore missing the second zinc finger of the DBD. This isoform inhibits oestradiol dependent transcriptional activation in a dominant negative fashion when it is co-transfected with the wild type ERα (Wang and Miksicek, 1991). Inuoe *et al* reported a mutated ERα with deleted exons 4 and 5, this also inhibited wild type ERα activity when they were co-transfected (Inoue *et al.*, 1996).

1.5 The Oestrogen Receptor beta (ERβ)

The intron/exon organisation of both the mouse and the human ERβ genes has been determined (Enmark *et al.*, 1997). The translated exons of the mouse ERβ gene span approximately 40kb which is considerably smaller than the ERα gene (Enmark, 1998). Multiple non-coding exons upstream (5') from exon 1 have also been identified (Enmark *et al.*, 1997; Peng *et al.*, 2003).

Homology between ERβ and ERα is particularly high in the DNA-binding domain (95% amino acid identity) and in the ligand binding domain (55% amino acid identity) (Enmark and Gustafsson, 1999) as shown in *Figure 1.5.*

Figure 1.5. Percentage amino acid homology between human ERα and ERβ.

1.5.1 ERβ Variants

Since the cloning of the human homologue of ERβ was first reported by Mosselman *et al* (1996) additional sequence information has become available and led to the identification of several variant isoforms of human ERβ (Inoue *et al.*, 2000; Lu *et al.*, 1998; Moore *et al.*, 1998; Ogawa *et al.*, 1998b; Ogawa *et al.*, 1998c). The molecular weights of the human ERβ variant proteins are given in *Table 1.1.*

Table 1.1. Human oestrogen receptor variants identified in the human for which there is evidence for protein expression. Taken from Scobie *et al.*, (2002).

Abbreviated name	Protein size (kDa)	References
Beta 1 "long" (wild type)	59.2	Ogawa *et al.*, 1998b
Beta 1 "short"	53.3 54.2	Mosselman *et al.*, 1996; Enmark *et al.*, 1997
Beta 2/cx "long"	55.5	Ogawa *et al.*, 1998c; Moore *et al.*, 1998
Beta 2/cx "short"	51	Ogawa *et al.*, 1998c
B1/B2 exon 5 deleted	36	Lu *et al.*, 1998; Inoue *et al.*, 2000
Beta 4	8 (?)	Moore *et al.*, 1998
Beta 5	7 (?)	Moore *et al.*, 1998

Bold indicates the ERβs studied in this thesis.

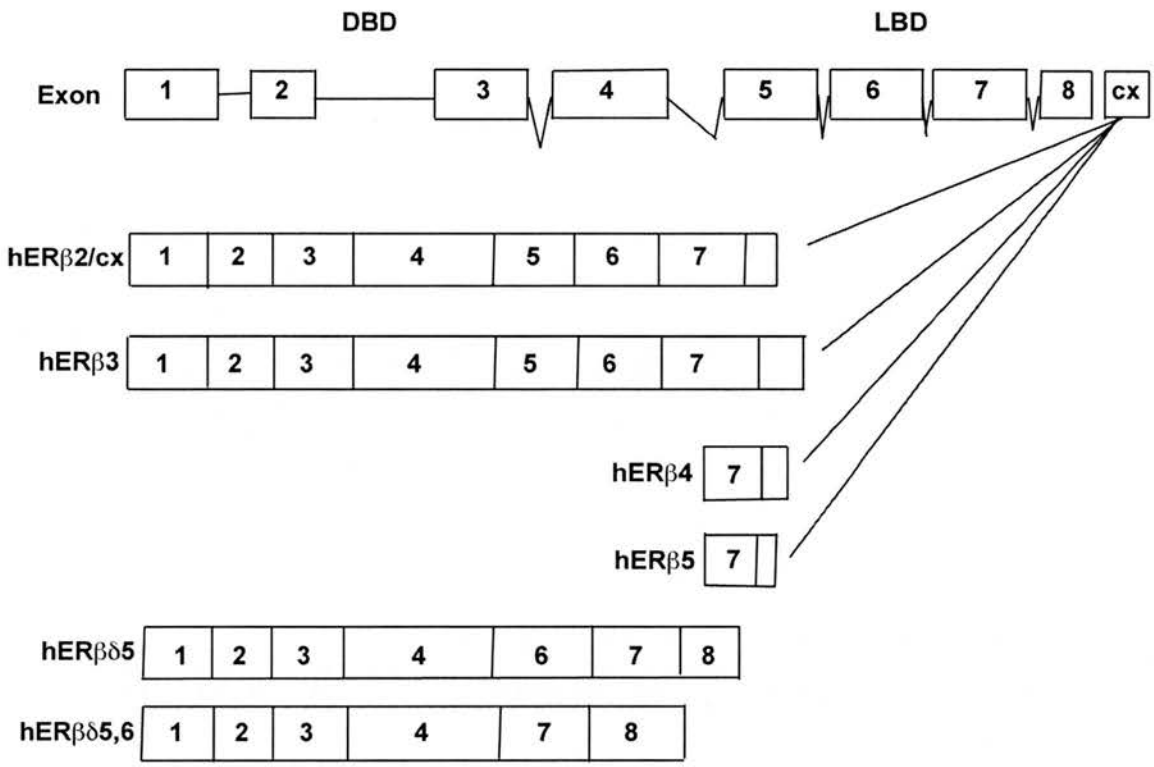
1.5.1.1 Alternate Start Sites

The original human ERβ cDNA published (X99101: Mosselman *et al.*, 1996) encoded a protein (~53kDa) that was eight amino acids shorter than the rat ERβ cDNA (Kuiper *et al.*, 1996). The human sequence was extended to include extra nucleotides and it was confirmed that it contained an ATG start site corresponding to the original rat clone (Enmark *et al.*, 1997). The size of the human ERβ protein was determined to be approximately 54.2kDa. The hERβ sequence was further extended to include a further 45 amino acids resulting in a protein size of 59.2 kDa (AB00659: Ogawa *et al.*, 1998b). Saunders *et al* have found that proteins corresponding to both the "long" and the "short" forms of ERβ can be detected on Western blots (Saunders *et al.*, 2002b and unpublished data).

1.5.1.2 Splice Variants and Loss of Exons

Several splice variants have been described for both the human (*Figure 1.6*) and rodent ERβ.

Figure 1.6. Illustration of the splice variants of human ERβ. Exons 1-8 are utilised in wild type ERβ (ERβ1) but the ERβ2 splice variant has exon 8 replaced by the alternative exon (exon 9) labelled cx. Adapted from Weihua *et al.*, (2003).



1.5.1.3 Identification of N- or C-terminal Variant Isoforms of ERβ

In 1998 a novel human ERβ variant was identified and named ERβ2 (also referred to as ERβcx) (Moore *et al.*, 1998; Ogawa *et al.*, 1998c). The open reading frame of the hERβ2 protein (55kDa) was found to be identical to ERβ1 from amino acids 1 to 468, the hERβ2 sequence then diverges at the C-terminus (see *Figure 1.6*). The last 61 C-terminal amino acids of ERβ1 are replaced by a unique 26 amino acid residue in ERβ2, due to the exchange of the last exon. ERβ2 therefore lacks some amino acids important for ligand binding and all of those that constitute the AF-2 core domain in helix 12 (Weihua *et al.*, 2003).

Moore *et al* have described five ERβ isoforms (ERβ1-5) detected by RT-PCR from human tissues (Moore *et al.*, 1998). The human ERβ3 is also identical to hERβ1 up to amino acid 468 and then diverges containing another 5 novel amino acids (see *Figure 1.6*). Therefore both the hERβ2 and hERβ3 variants are truncated at the C-terminus, have a disrupted helix 11, and are missing helix 12. They are therefore are unable to bind ligand or have AF-2 mediated transcriptional activity (Peng *et al.*, 2003).

Human ERβ5 mRNA has been found to contain a sequence between exon 7 and part of exon 9 which are not present in either hERβ1 or hERβ2 mRNA (Moore *et al.*, 1998; Peng *et al.*, 2003). These specific ERβ5 mRNA sequences can be found immediately following exon 7 sequences of intron 7 of the human ERβ gene, 28 nucleotides downstream of those present in hERβ2. Thus suggesting that a cryptic splice acceptor is present within exon 9 and that the normal splice donor site is not recognised (Peng *et al.*, 2003).

RT-PCR using specific primers has detected mRNA specific for ERβ2 in multiple tissues (Moore *et al.*, 1998; Scobie *et al.*, 2002). This isoform seems to be particularly abundant in testis (Makinen *et al.*, 2001; Moore *et al.*, 1998). The ERβ4 and ERβ5 mRNAs have also been detected in the testis and ovary as well as in several other tissues and human cell lines (Moore *et al.*, 1998). ERβ4 has generally been found to be expressed to a lesser extent than ERβ5 except within the testis (Moore *et al.*, 1998; Scobie *et al.*, 2002).

1.5.1.4 Loss of Exons

Lu *et al* detected ERβ mRNAs lacking exons 5 (ERβΔ5) and/or 6 (ERβΔ5/6) in human breast, ovary, uterus and breast cancer by RT-PCR with primers located in exons 4 and 8 (Lu *et al.*, 1998). Messenger RNA for the exon 5 deleted form of ERβ has also been identified in a breast cancer cell line using RT-PCR and RNase protection assays (Vladusic *et al.*, 1998) and isolated from human testis cDNAs (Inoue *et al.*, 2000). Exon 5 deletion results in a frame shift in the reading frame of the protein and the introduction of five alternative amino acids followed by a stop codon (Inoue *et al.*, 2000).

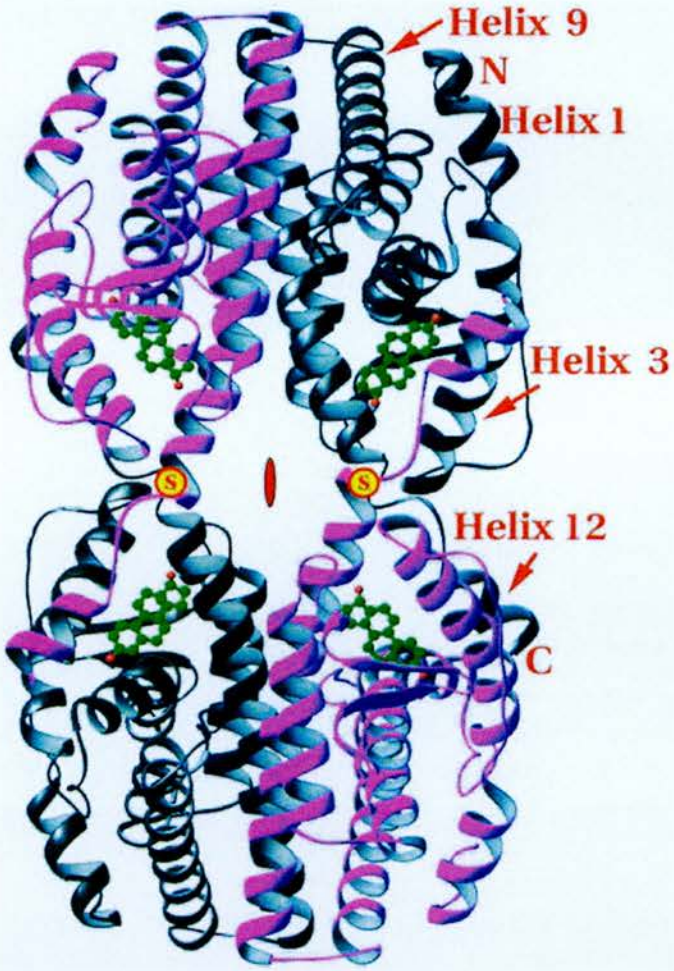
Isoforms of rat ERβ lacking exon 3 (Price *et al.*, 2001) and isoforms containing an insertion of 54 nucleotides encoding an additional 18 amino acids between exons 5

and 6 (Chu and Fuller, 1997) have both been documented to modulate gene transcriptional activation by ERα and the ERβ wild type. However these splice variants have not been detected in human (Chu and Fuller, 1997; Chu *et al.*, 2000).

1.6 Oestrogen Receptor Ligand Binding Domain

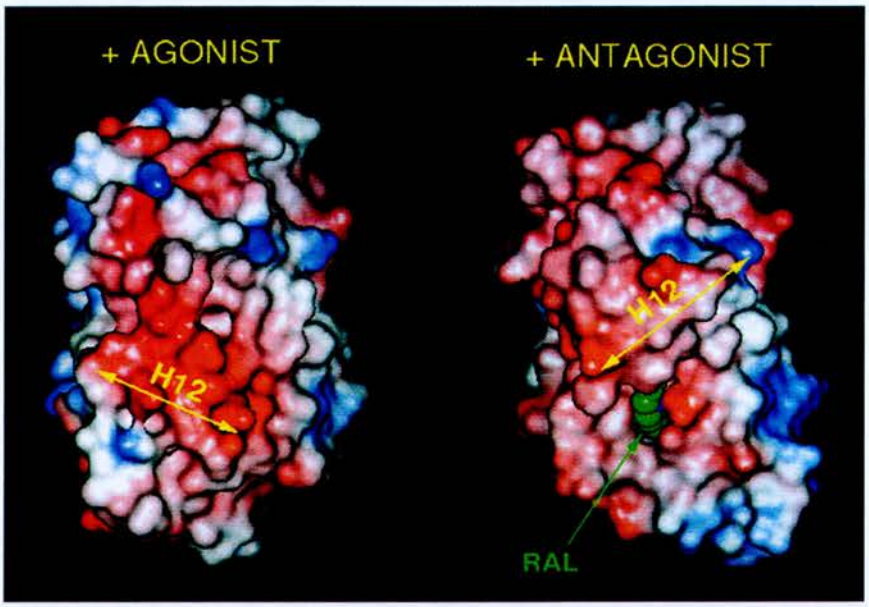
The ligand binding domain (E domain) mediates ligand binding, receptor dimerisation, nuclear translocation and transactivation of target gene expression. The LBD shares 53% homology between ERα and ERβ (Weihua *et al.*, 2003). Crystallographic studies have shown that the overall tertiary structure of the ligand-binding domain of human ERα (Brzozowski *et al.*, 1997) and ERβ (Pike *et al.*, 1999) are similar and contain 12 helices (*Figure 1.7*). Studies in the rat ERβ have shown that there are key amino acids within the ligand binding domain which are conserved between the ER subtypes and that amino acids surrounding the LBD cavity are very similar (Kuiper *et al.*, 1997). Alignment of the E domain sequences of human ERα and ERβ has revealed two regions of the protein with significant homology; hERβ 260-343/hERα 353-431, and hERβ 418-453/hERα 511-547 (Enmark *et al.*, 1997) which include helix 12 (*Figure 1.7*). Therefore it is not surprising that some oestrogenic compounds that have been tested bind to rat ERα and ERβ with similar affinities (Kuiper *et al.*, 1998) or have similar potencies in activation of ERE reporter mediated gene transcription.

Figure 1.7. Schematic representation of the hERα, illustrating the helices upon receptor dimerisation. Taken from Tanenbaum *et al.*, (1998).



Agonists induce a conformational change in ERα so that the AF-2 core helix (helix 12) completes the formation of a hydrophobic groove (Nilsson *et al.*, 2001) (*Figures 1.7* & *1.8*) which is important in binding to LXXLL motifs (NR box) found in p160 co-activator proteins (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998). When ERα is bound to an antagonist, helix 12 is displaced from this position and instead occupies the hydrophobic co-activator binding groove, formed by helices 3, 4, and 5, therefore precluding co-activator binding (Brzozowski *et al.*, 1997; Kraichely *et al.*, 2000; Shiau *et al.*, 1998). The conformations adopted by the receptor following agonist and antagonist binding are illustrated in *Figure 1.8*. The position adopted by helix 12 following ligand binding is therefore one indicator of the ability of nuclear receptors to recruit co-activators.

Figure 1.8. Diagram to show the receptor conformation upon ligand binding with agonist or antagonists (eg.raloxifene). Taken from Nilsson *et al.*, (2001).



1.6.1 Importance of the AF-2 Domain

The ligand dependent transactivation domain AF-2 is of particular interest because it provides a mechanistic explanation for the observed differences between agonism and antagonism of the ER. The AF-2 recruits co-factors upon ligand binding and it is these proteins which act to inhibit or increase transcriptional activity by binding with the pre-initiation complex. The ERβ2 lacks the AF-2 domain and part of the ligand binding domain, therefore it is unable to bind correctly to ligands or to recruit co-factors.

The AF-2 is part of the LBD and the conformation of the LBD has been shown to be of vital importance for AF-2 function. The AF-2 region was initially identified in the mouse ERα where detection analysis demonstrated a large segment of the LBD to be important for ligand binding (Lees *et al.*, 1990). Mutation and deletion studies of LBDs revealed a conserved segment in the very C-terminus of the LBD that was shown to be essential for ligand dependent activation of transcription (reviewed in Warnmark *et al.*, 2003). This conserved segment has been shown by crystallographical studies to be an amphipathic helix (helix 12) within the ER. This helix projects away from the core structure whereas in a ligand-bound ER, helix 12 is folded up against the core creating a lid over the ligand binding pocket. This

amphipathic, α-helical structure is critical for AF-2 function and mutagenesis studies suggest that co-activator interactions with ER may depend on the integrity of this activation region (Danielian *et al.*, 1992). Therefore, structural data together with transcriptional activation data imply that the positioning of helix 12 is crucial for receptor activation.

There is a conserved tyrosine residue located immediately before the AF-2 domain (White *et al.*, 1997). This residue is conserved in the ER sequence of every species examined to date (Smith, 1998). The importance of this tyrosine, Tyr537, in human ERα (Tyr541 in mouse ERα) for ER function is highlighted by site-directed mutagenesis studies in which amino acid substitutions (serine or alanine for tryosine 537) in human ERα resulted in a constitutively active ERα mutant (Weis *et al.*, 1996). In contrast to wild-type ERα, the mutant receptor interacts with co-activators to a lesser extent in the absence of hormone (Weis *et al.*, 1996). Therefore data has demonstrated that the conserved tyrosine (Tyr537) and the adjacent amphiathic helix form a conformation-dependent interaction surface for co-activators and that this region is important for ER transcriptional activity.

1.6.2 Oestrogen Receptor Dimerisation

Dimerisation of the ER upon ligand binding is necessary for DNA binding and hence gene transcription. Transcriptional activity of the dimer complex has been found to be dependent on a functionally intact AF-2 region within both ERα and ERβ1 (Warnmark *et al.*, 2003). The complex is able to initiate transcription when only one ER subunit interacts with a ligand, albeit to a lower extent than if both subunits interacted with a ligand (Pettersson *et al.*, 1997).

1.6.2.1 Domains Involved in Dimerisation

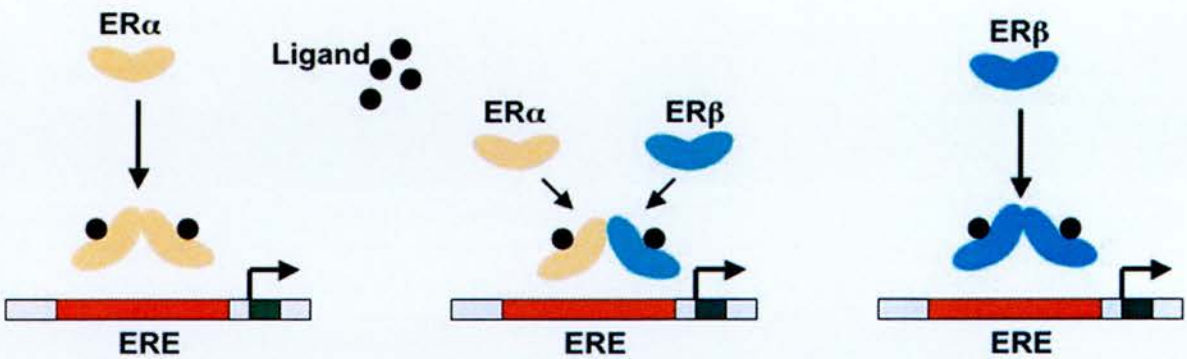
Mutations that interfere with the dimerisation of steroid receptors generally result in receptors that are transcriptionally inactive (Tamrazi *et al.*, 2002). Human ERα and hERβ have almost identical DBDs and the regions of the LBDs involved in dimerisation also share a significant degree of similarity (Pettersson and Gustafsson, 2001). The region of the second zinc finger within the DBD is thought to contribute to specific receptor dimerisation via the CPATNQC sequence and this sequence is identical in hERα and hERβ (Pettersson and Gustafsson, 2001). Residues 507-514 have been identified as vital for dimerisation activity of mouse

ERα and are largely conserved in rodent and human ERβ (Ekena *et al.*, 1997; Fawell *et al.*, 1990; Lees *et al.*, 1990).

1.6.2.2 Homodimers and Heterodimers

A variety of techniques have been used to demonstrate the ability of ERα and ERβ to form homodimers (ERα/ERα or ERβ/ERβ) and heterodimers (ERα/ERβ) (*Figure 1.9*). These include gel shift assays (Cowley *et al.*, 1997), GST pull down assays (Cowley *et al.*, 1997; Ogawa *et al.*, 1998b; Pettersson *et al.*, 1997), immunoprecipitation (Pettersson *et al.*, 1997) and a mammalian two hybrid system (Pettersson *et al.*, 1997).

Figure 1.9. Diagram to demonstrate ER homodimerisation and heterodimerisation.



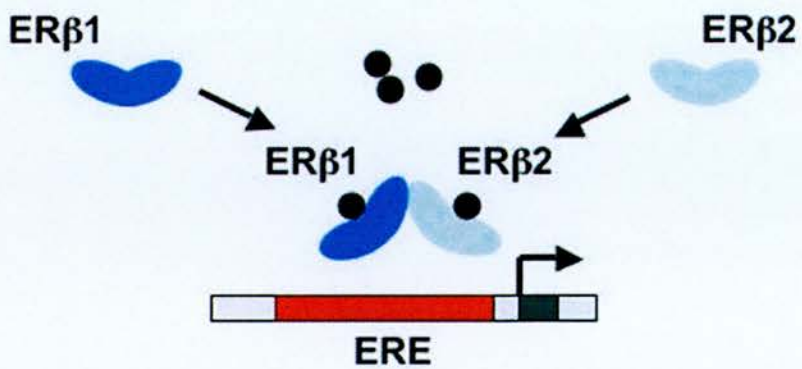
Heterodimer formation between hERα and ERβ means that *in vivo* they may cooperate in regulating oestrogen-responsive gene expression in cell types in which they are co-expressed (Pettersson *et al.*, 1997). Co-expression of ERα and ERβ results in the preferential formation of receptor heterodimers, instead of ERα and especially ERβ homodimers in vitro (Enmark and Gustafsson, 1999; Hall and McDonnell, 1999). In binding assays, human ERα/ERβ heterodimers bind to the consensus ERE with an affinity similar to that of ERα homodimer (2nM K_d) and is greater than that of the ERβ homodimer (~8nM K_d) (Cowley *et al.*, 1997). Similar results were observed in transient transfections in human cell lines (Hall and McDonnell, 1999; Pettersson *et al.*, 1997).

The molecular basis for the reduced DNA binding activity of ERβ/ERβ homodimers is unclear. Differences within the DBD of the two receptors are unlikely to account

for the variation since they differ by only two residues, neither of which seems to be in a position that is likely to affect their DNA binding properties. An alternative possibility is that the receptors differ in their ability to dimerise. It seems likely that the residues required to form the homodimer interface in ERα and ERβ dimers are distinct. Although precise dimerisation interfaces in these two receptors have yet to be identified, functional analysis of a series of mutations in helix 10 of ERα indicate that the residues are similar but not identical to those required for ERα/ERβ heterodimerisation (Cowley *et al.*, 1997).

The truncated ERβ2 splice variant contains the DBD and most of the LBD including the conserved dimerisation sequence. It is able to form heterodimers with ERα or with ERβ1 in gel shift assays (Cowley *et al.*, 1997) and in GST Pull down assays (Ogawa *et al.*, 1998c) (as illustrated in *Figure 1.10* with ERβ1). ERβ2 shows preferential heterodimerisation with ERα rather than with ERβ1 and therefore may act to inhibit ERα's binding to DNA (Weihua *et al.*, 2003).

Figure 1.10. Diagram to demonstrate ERβ1/ERβ2 heterodimerisation.



1.7 DNA Binding and Gene Activation

Gene activation occurs once the ligand has bound and homo- or heterodimers have formed. In the classical pathway, the receptors bind to an oestrogen responsive element (ERE) on the DNA and activate transcription of the target gene.

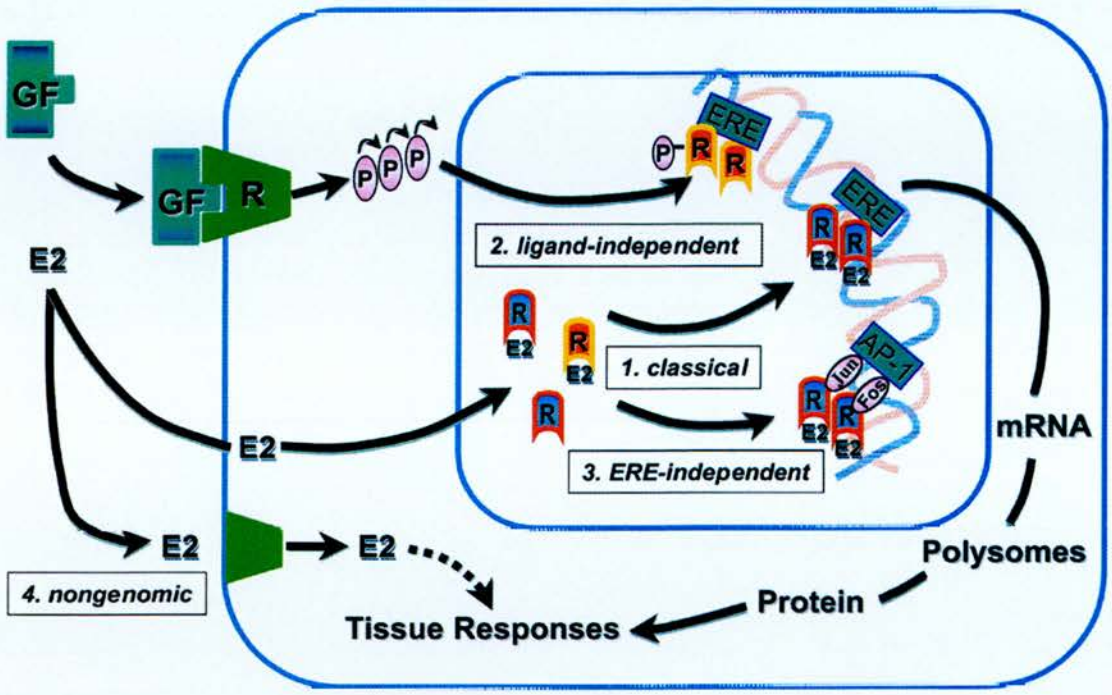
1.7.1 Structure of the DNA Binding Domain

The most conserved region of the ERα and ERβ isoforms is the DNA binding domain (DBD) in the C functional region (96% homology). The DBD contains two functionally distinct zinc fingers co-ordinated by eight highly conserved cysteine residues which are involved in ERE sequence recognition and dimerisation of the ER DNA binding domains (Loven *et al.*, 2001; Scobie *et al.*, 2002). Amino acids near the C-terminus of the first zinc finger in the P-box determine the half-site ERE sequence to which the receptor binds while the amino acids in the D-box (near the terminus of the second zinc finger) provide important protein-protein contacts in the receptor dimers (Pace *et al.*, 1997). Each ER monomer is bound to DNA in the major groove with the ER dimer located predominantly on one face of the DNA helix (Klinge, 2001). Outside the DNA binding domain, specific contacts between the ER dimer and the sugar phosphate backbone of the ERE play a role in sequence recognition and high affinity binding. These regions are highly conserved between the ERα and ERβ isoforms, particularly the P and D-boxes (Vanacker *et al.*, 1999) and are critical for target DNA recognition and specificity (Loven *et al.*, 2001). Therefore it is not surprising that both receptors bind to similar EREs (Nilsson *et al.*, 2001).

1.8 Activation Pathways

Oestrogen receptors can regulate gene transcription via four pathways as shown in *Figure 1.11* (Hall *et al.*, 2001). ERs can either bind directly to the promoter of target genes (EREs) in the classical pathway (1) or by binding indirectly through an ERE independent mechanism involving other transcription factors such as Sp1, NFκB or AP-1 (2) (Nilsson *et al.*, 2001; Shang and Brown, 2002). There is also a ligand independent activation mechanism involving phosphorylation of the ERs via a phosphorylation cascade (3) and a non-genomic pathway (4).

Figure 1.11. The multifaceted mechanisms of oestradiol and ER signalling. Taken from Hall *et al.*, (2001).



1.8.1 Classical Gene Activation (ERE)

In contrast to the wide variety of actions that oestrogens have in various organs, tissues and cells, relatively few genes have been defined that are directly regulated by oestrogen treatment. Using ER-binding fragments isolated from human genomic DNA, Northern blot analysis and screening, ER binding fragments were found to contain consensus oestrogen binding elements (EREs) (Inoue *et al.*, 1993). The ERE sequences were identified by aligning sequences with shared homologies in the 5' flanking regions of the oestrogen regulated vitellogenin genes A1, A2, B1 and B2 from *Xenopus laevis* and the chicken apo-VLDLII gene (Walker *et al.*, 1984). Since then it has been demonstrated that the minimal consensus ERE sequence is a 13 base pair palindromic inverted repeat; 5-GGTCAnnnTGACC-3' where "n" is any nucleotide (Klinge *et al.*, 1997; Klinge *et al.*, 2001). However, most oestrogen regulated genes contain imperfect, non-palindromic EREs (Driscoll *et al.*, 1998).

In her review Klinge listed 38 oestrogen-responsive genes whose promoters contain functional EREs (Klinge, 2001), for example those encoding *Xenopus* vitellogenin A1 and B1, chicken apo VLDII, and human genes including pS2, oxytocin, c-*fos*, c-*myc*, TGF-α, lactoferrin, prolactin, the progesterone receptor, cathsepin D and

complement 3 (Inoue *et al.*, 1993; Klinge, 2001). Some genes, for example the *Xenopus* vitellogenin A2 (referred to here as Vit ERE), and the human oxytocin genes, contain a single perfect or sequence variant copy of the consensus ERE. Other oestrogen responsive genes, for example *Xenopus* vitellogenin B1, ovalbumin, prolactin, the rat progesterone receptor and human c-fos contain multiple copies of the ERE but not the palindrome. These genes usually have one or more base changes compared to the consensus ERE and function synergistically to induce oestradiol dependent gene activation (Klinge *et al.*, 1997; Sathya *et al.*, 1997). It has been documented that differences in ERE sequence impact on ER binding affinity and transcriptional activation (Klinge, 2001). It has been demonstrated that if the ERE sequence deviates from the consensus sequence by even a single base pair, the receptor exhibits a reduced affinity for the ERE and a decreased transcriptional activity (Wood *et al.*, 2001).

The change of one nucleotide in the ERE can determine the formation of a different interconnected hydrogen bond network. This suggests that each ERE sequence can induce a unique conformational change in the DBD structure (Wood *et al.*, 1998) and may induce different transcriptional responses. The orientation of a consensus or imperfect ERE relative to the TATA sequence within the DNA can have profound effects on the expression of an oestrogen responsive reporter plasmid when it is transfected in MCF-7 cells (Sathya *et al.*, 1997). For example, location of single or tandem copies of ERE sequences upstream of the luciferase gene promoter was demonstrated to be more effective in inducing reporter expression than if it were located downstream (Sathya *et al.*, 1997).

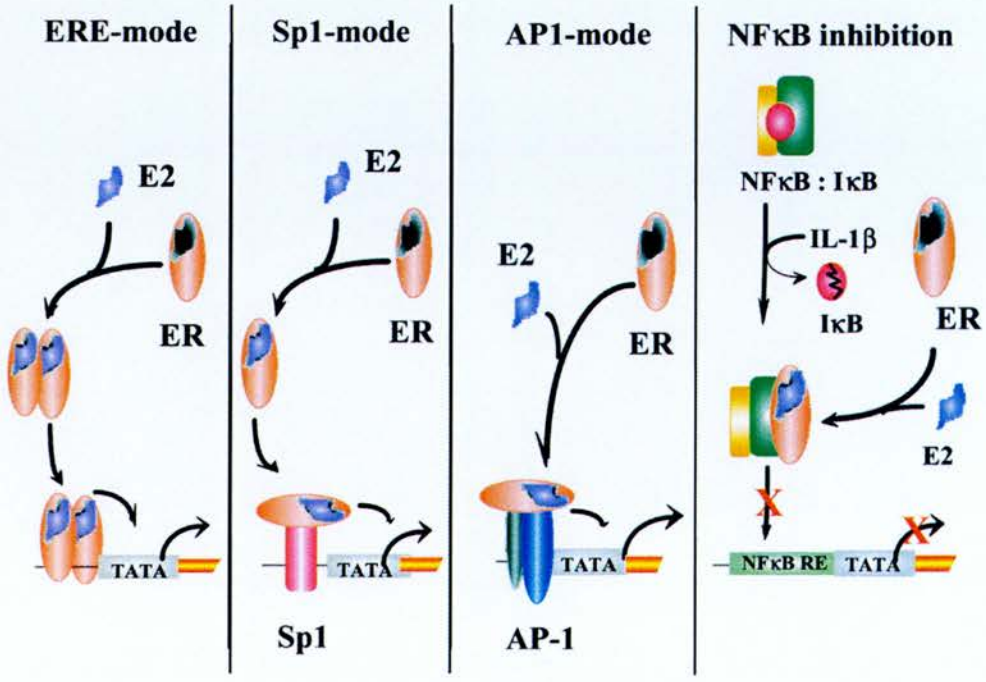
Using gel mobility shift, DNase I footprinting and methylation interference assays it has been demonstrated that the DBD bound only as a dimer to the A2 ERE (Wood *et al.*, 1998). Spacing between EREs is also important because when the ER dimer binds to an ERE a conformational change is induced within the DNA resulting in a bend of 65°. The helical separation between the EREs and the TATA box may influence the bending induced and therefore the interaction of transcription factors with the TATA box (Nardulli *et al.*, 1996). Conformational changes vary for different EREs. For example, the Vit ERE maximally activates transcription when it is separated from the TATA sequence by 2.6 or 3.6 helical turns, whereas the pS2 ERE maximally activates transcription when it is separated by 3 helical turns (Wood *et al.*, 1998).

The length and spacing of the ERE palindrome is also critical for high affinity ER-ERE binding. ERα does not bind to ERE half sites in which the palindrome is separated by 2, 4 or 5 base pairs (Klinge, 2001). It has been argued that the minimal length of the ERE containing sequence should be considered to consist of 15 base pairs (Klinge, 2001) and not 13 as reported previously (Klein-Hitpass *et al.*, 1988). Extension of the ERE thus resulting in a 17 or 19 base pair sequence did not further increase affinity for either ERα or ERβ (Klinge, 2001). The consensus *Xenopus* vitellogenin A2 ERE palindrome is 19 base pairs in length.

1.8.2 ERE-independent Gene Activation

For many years it was considered that the only mechanism through which the oestrogen receptors affected transcription of oestrogen sensitive genes was by direct binding of the ERs to EREs. However, it is now understood that ERα and ERβ1 can activate gene expression through alternative, non-classical pathways as pictured in *Figures 1.11* & *1.12*.

Figure 1.12. Models representing various modes through which ERs can modulate transcription of genes. Taken from Nilsson *et al.*, (2001).



Both ERα and ERβ1 can interact with the transcription factors Fos and Jun, influencing their function at AP-1 sites to stimulate gene transcription (as shown in

Figures 1.11 & 1.12) (Olefsky, 2001; Paech *et al.*, 1997). ER action can proceed through the AP-1 sites via two pathways; (i) ERα acts through an activation function dependent pathway, which does not require the DBD but does rely on an intact AF-1 or AF-2 and allows activation with agonists (Price *et al.*, 2001), (ii) ERβ1 activates AP-1 responsive transcription through an AF-1 independent pathway that requires an intact DBD and allows activation of AP-1 sites with ER antagonists. Hence, in the presence of ERα, agonists such as E_2 and DES, as well as the antagonist tamoxifen, function as agonists in the AP-1 pathway. In the presence of ERβ1, raloxifene and tamoxifen act as full agonists in the AP-1 pathway whilst E_2 acts as an antagonist (Paech *et al.*, 1997). The ERβ DBD sequesters co-repressors from the AP-1 transcription complex, releasing it from the suppression and resulting in AP-1 dependent transcription (Webb *et al.*, 1999).

Several well known gene promoters are regulated by AP-1 sites and in opposite directions by ERα and ERβ1 (Cowley *et al.*, 1997; Mosselman *et al.*, 1996; Pettersson *et al.*, 1997). For example, on the AP-1 site of the collagenase promoter, E_2 elicits transcriptional activation via ERα but represses it with ERβ1 (Ogawa *et al.*, 1998c). Therefore, ERα and ERβ have been shown to respond differently to certain ligands at an AP-1 element demonstrating the different regulatory functions for the two ER isoforms.

ERα and ERβ can also modulate the expression of genes without directly binding to DNA. One example is the interaction between ERα and the c-*rel* subunit of NFκB complex (Nilsson *et al.*, 2001) as pictured in *Figure 1.12*. Genes containing GC-rich promoter sequences can be activated via an ERα-Sp1 complex (Hall *et al.*, 2001) in a ligand independent manner (Nilsson *et al.*, 2001) (*Figure 1.12*).

1.9 Importance of the AF-1 Domain

The N-terminal A/B domain of the ER includes a ligand-independent activation function (AF-1) (Kraus *et al.*, 1995; McInerney *et al.*, 1998b). However, ERα and ERβ have little or no sequence homology within their A/B domains. Comparison of the functional activity of the AF-1 domains of the two ERs has revealed that in ERα, the domain is very active in stimulating reporter gene activation of target gene expression from a variety of EREs in different cell lines (Cowley and Parker, 1999). However the activity of the AF-1 domain in ERβ1 is negligible under the same

conditions. Anti-oestrogen agonism has been reported to be via the AF-1 region of ERα and is not supported by AF-1 region of ERβ1 (McInerney *et al.*, 1998b). Therefore these differences in the amino-terminal regions of ERα and ERβ contribute to the cell and promoter specific differences in transcriptional activity of the receptors and their ability to respond to different ligands, providing a mechanism for differentially regulated transcription by these two ERs (McInerney *et al.*, 1998b).

Human ERα contains serine residues within the AF-1 region which become phosphorylated with ligand binding (Weis *et al.*, 1996) as well as being regulated by phosphorylation via kinase signalling pathways (Sadovsky *et al.*, 1995). Mouse ERβ1 has been found to contain serine residues also within the AF-1 (Tremblay *et al.*, 1999b), but with slightly different positions compared to hERα.

1.10 Co-factors

To regulate transcription, ligand bound ERs interact with the general transcriptional machinery and form the transcription pre-initiation complex at the transcription start site of the target gene. This can be achieved either by direct contact between the ER and the transcription factors, or by means of co-activators (Beato and Klug, 2000).

Ligand binding of ERα or ERβ induces a conformational change in the structure of the protein resulting in the recruitment of interacting proteins (co-activators or co-repressors) (*Figures 1.13* & *1.14*) that can have a profound effect on ER-mediated gene transcription (reviewed in Klinge, 2000). Ligand binding induces a change in the position of helix 12, next to the AF-2 domain and results in the exposure of critical residues required for co-activator recruitment (Klinge, 2000; Pike *et al.*, 1999; Routledge *et al.*, 2000; Warnmark *et al.*, 2001). There are differences between the conformational change in ERα and ERβ1 upon ligand binding which therefore lead to variations in recruitment of co-factors.

Figure 1.13. Schematic diagram to show the role of co-activators. Taken from Lonard and Smith (2002).

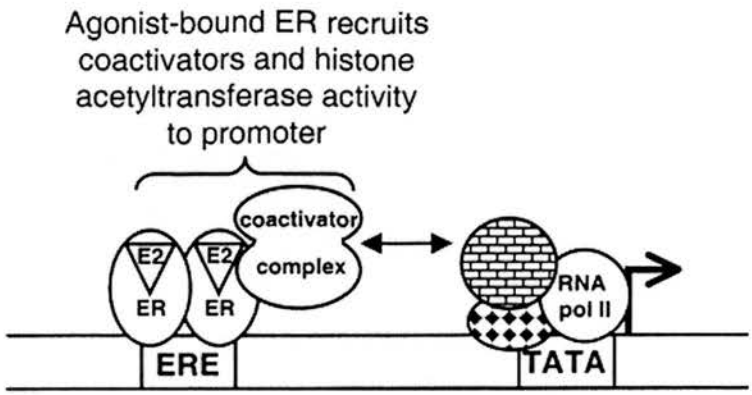
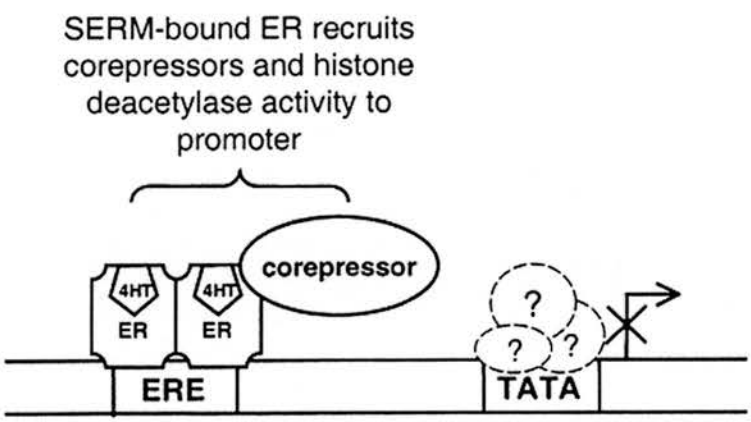


Figure 1.14. Schematic diagram to show the role of co-repressors. Taken from Lonard and Smith (2002).



It has been shown that the ER interacts with a number of nuclear proteins *in vitro*, some of which have functional consequences for transcriptional activation (Klinge, 2000). Over the last few years at least nineteen different receptor interacting proteins (co-factor proteins) have been identified (Klinge, 2000). These include SRC-1/NCoA1 (Onate *et al.*, 1995), GRIP-1/TIF-2/NCoA2 (Voegel *et al.*, 1996), p/CIP/AIB1/ACTR (Anzick *et al.*, 1997) and CBP/p300 (Chrivia *et al.*, 1993) (reviewed in McKenna *et al.*, 1999).

Co-activators act by turning on target gene transcription (*Figure 1.13*) whilst co-repressors act to turn off activated target genes (*Figure 1.14*). The majority of these co-factors exist as multi-protein complexes which upon binding to the LBD of the receptor, bridge the ER to either chromatin compartments such as histones or to components of the basal transcription machinery, or to both.

Co-activators interact with the agonist bound LBD and AF-2 interaction surface of the receptor dimers (Feng *et al.*, 1998) via a conserved region known as the NR box (Heery *et al.*, 1997; Onate *et al.*, 1998). Domain-mapping experiments reveal that a region (amino acid 535 to 554) that contains helix 12 (amino acid 538 to 546) in the crystal structure of the ERα LBD (Brzozowski *et al.*, 1997) is required for SRC recruitment. The co-activator NR box consists of three conserved motifs each consisting of LXXLL, where L is leucine and X is any amino acid (Heery *et al.*, 1997). The LXXLL motif forms an amphipathic α-helix that binds a hydrophobic cleft formed in the LBDs of ERs after ligand binding (Shiau *et al.*, 1998). It has been demonstrated that the central motif 2 is sufficient for *in vitro* binding to the ER. However for the optimal potentiation of ER-mediated transcription in transiently transfected cells three motifs are required (Kalkhoven *et al.*, 1998).

The length and orientation of these helical motifs in the co-activators are important for high-affinity interaction with the receptor's AF-2 domain (Nolte *et al.*, 1998). The recruitment of SRCs depends not only on their LXXLL motif but also the adjacent basic residues, especially those in the C-terminal region surrounding the hydrophobic groove which are involved in the recognition of charged residues in the receptor's LBD (Chang *et al.*, 1999; Mak *et al.*, 1999; McInerney *et al.*, 1998a). Thereafter specific hydrophobic and electrostatic interactions between the motif and the receptor occur, resulting in stable interactions of the co-activator with the receptor. Not all co-activator LXXLL motifs are identical and receptor binding selectivity can be achieved by altering sequences flanking the LXXLL core motif (Chang *et al.*, 1999). To add to the complexity of the receptor co-activator complexes, different ligands induce different helix 12 conformations and therefore alter the ER's ability to recruit these SRC NR boxes (McInerney *et al.*, 1998a; Torchia *et al.*, 1997).

1.10.1 The Steroid Receptor Co-activator (SRC)

The SRC was the first steroid co-activator to be identified (Onate *et al.*, 1995). Once bound to the ER, co-activators such as the SRC are thought to enhance NR-based transcription by several mechanisms (Jenster, 1998). Many co-activators including SRC possess intrinsic histone acetylase activity (HAT) suggesting that chromatin remodelling plays a role in transactivation. The HAT domain in SRC was mapped out to the C terminus of the protein, between amino acids 1107 and 1441 (Spencer *et al.*, 1997). SRC HAT activity may not be essential for ER directed initiation of transcription as site-directed mutations to the C-terminal region do not significantly affect its co-activation function (Spencer *et al.*, 1997). Although SRC may play a role in chromatin remodelling and the assembly of general transcription factors through direct and indirect recruitment of other co-activators (Leo and Chen, 2000).

There are three isoforms of the SRC (SRC-1, -2, -3) (Xu and O'Malley, 2002) all of which interact with the ERs and activate transcription. SRC-1 gene expression has been identified in the testes as well as in other oestrogen target tissues (Xu *et al.*, 1998). SRC-1 knockout mice have been shown to exhibit nearly normal male and female reproductive behaviours and fertility (Apostolakis *et al.*, 2002; Xu *et al.*, 1998). However, oestrogen target organs such as uterus, prostate, testes and mammary gland exhibited decreased growth and development in response to steroid hormones (Xu *et al.*, 1998). The SRC-1 knockout model may not reveal the full activity of SRC-1 as it has been documented that SRC-2 and SRC-3 proteins can compensate for the loss of SRC-1 (Xu and O'Malley, 2002; Xu *et al.*, 1998).

1.10.2 Other Co-activators and Co-repressors

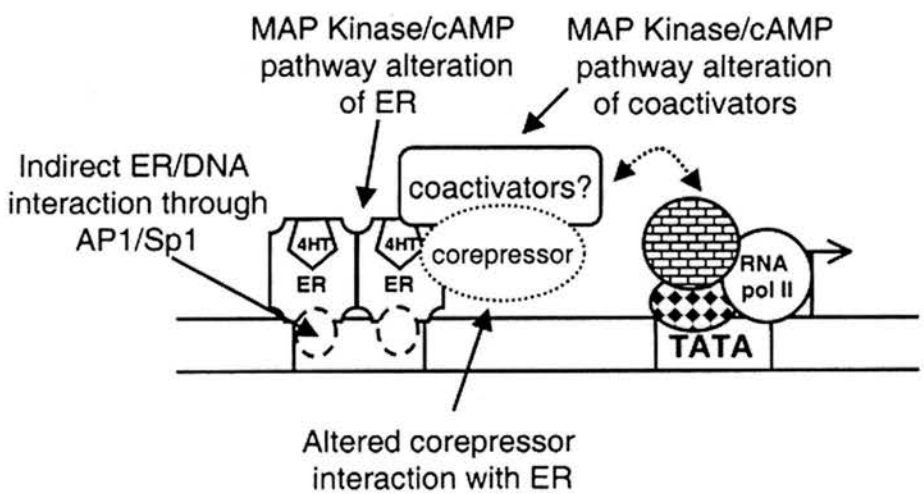
There are several other co-activators involved in nuclear receptor activation, including CBP/p300 complexes, which are viewed as general co-activators and co-integrators involved in multiple signalling pathways. They are ubiquitously expressed, possess acetyltransferases activity and contain docking sites for ERs via N-terminal NR-boxes (Nilsson *et al.*, 2001). The TRAP/DRIP is another co-activator complex which is thought to connect ERs directly to the basal transcription machinery (Kang *et al.*, 2002). ERα is less effective than ERβ in recruiting the TRAP/DRIP complex *in vitro* (Montano *et al.*, 1997). The RIP-140 co-repressor interacts with the AF-2 domain, but exhibits inhibitory coregulatory functions

because it can antagonise SRC-1 *in vivo* and compete for binding to the AF-2 (Treuter *et al.*, 1998).

1.11 Activation of Steroid Receptors via a Ligand Independent Pathway

All of the steps involved in transcriptional activation of ER-dependent genes, i.e. ligand binding, ER dimerisation, DNA binding, and the interaction with co-activators appear to be influenced by phosphorylation of the ER (Nilsson *et al.*, 2001). In the absence of ligand, alternative signalling pathways can also modulate ERs through phosphorylation. These alternative pathways include regulators of the general cellular phosphorylation state, such as mitogen activated protein kinase (MAP kinase) (*Figure 1.15*) protein kinase A (PKA), or protein kinase C (PKC) (Ignar-Trowbridge *et al.*, 1996; Ince *et al.*, 1994).

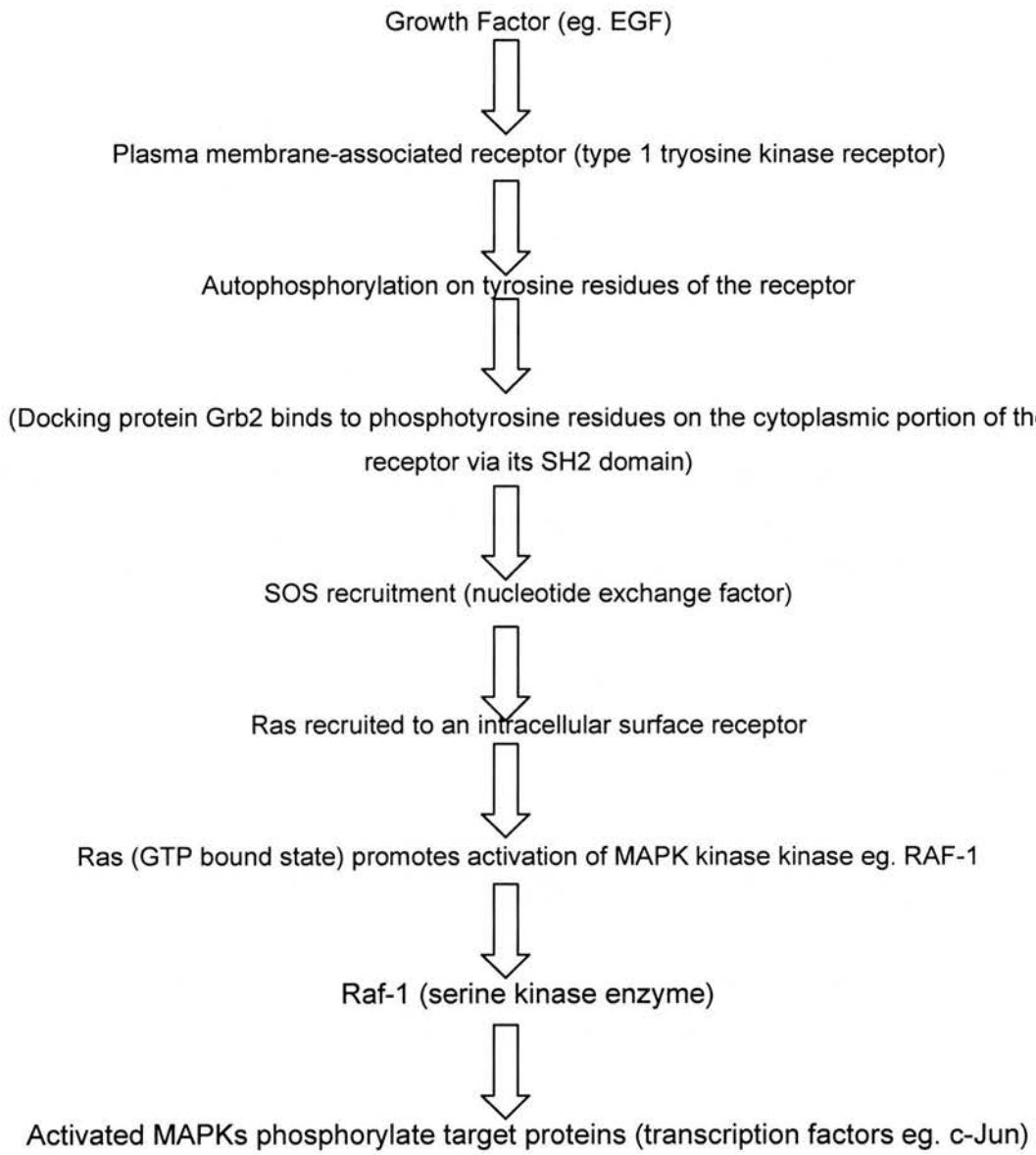
Figure 1.15. Schematic diagram to show activation of gene transcription through the MAP kinase pathway. Taken from Lonard and Smith (2002).



1.11.1 The MAP Kinase Signalling Cascade

MAP kinases are activated through several distinct signalling pathways, one of which is mediated through tyrosine kinase-type cell membrane receptors activated by growth factors such as EGF, IGF-1 and TNF-α (Kato *et al.*, 2000). Upon binding of a growth factor to the receptor extracellular's domain, the receptor undergoes autophosphorylation on tyrosine residues and subsequently acquires the potential to activate a number of intracellular enzymatic activities, as schematically represented in *Figure 1.16* (reviewed in Smith 1998).

Figure 1.16. A schematic representation of the MAP Kinase signalling pathway, adapted from Smith (1998).

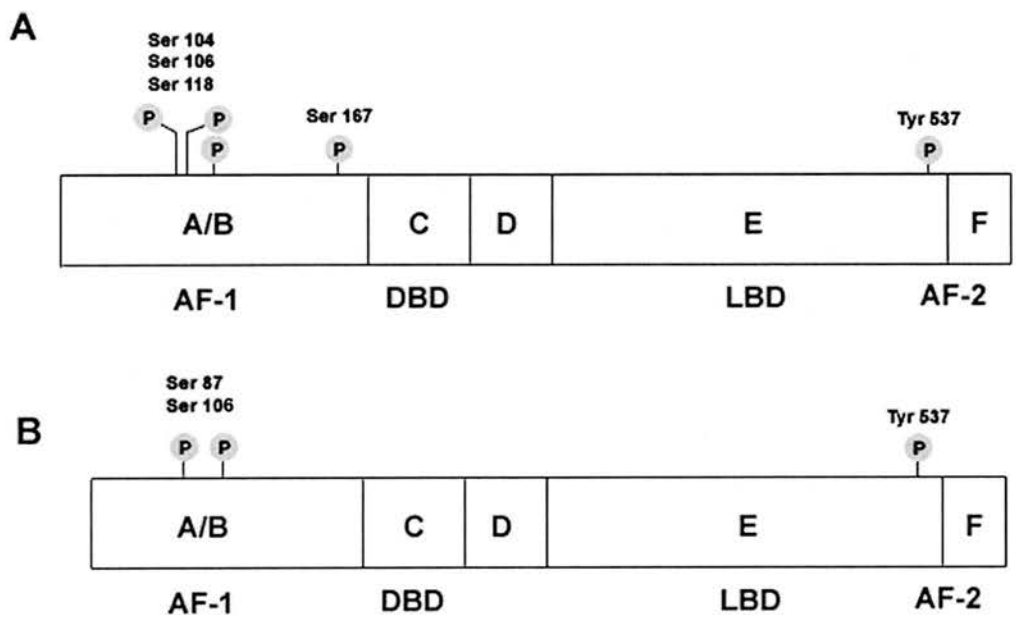


1.11.2 Phosphorylation of the Oestrogen Receptor

All steroid receptors including ER are phosphorylated after binding their respective ligands (reviewed in Weigel and Zhang, 1998). In addition, ERα and ERβ can be phosphorylated and activated in the absence of ligand binding (Tremblay *et al.*, 1999b; White *et al.*, 1997).

Using a combination of direct (amino acid and radiolabel sequencing) and indirect (deletion and site-directed mutagensis) approaches, five phosphorylation sites have been mapped within the human ER (*Figure 1.17*). Four of these sites (Ser104, Ser106, Ser118 and Ser107) are located in the A/B domain while the fifth site (tyrosine 537) is located within the LBD (reviewed in Smith, 1998).

Figure 1.17. Full length hERα is composed of 595 amino acids, with five known phosphorylation sites (A). Adapted from Smith (1998). Full length hERβ1 is composed of 530 amino acids with three known phosphorylation sites (B).



The AF-1 region within the ER's N-terminus contains several conserved serine residues which are targets for phosphorylation. Deletion mapping and mutagenesis of human ERα have revealed that phosphorylation of Serine 118 is required for full activity of AF-1 (Kato *et al.*, 1995). Phosphorylation of Ser118 in human ERα is induced by growth factors such as the epidermal growth factor (EGF) and is dependent on the MAP kinase (mitogen activated protein kinase) pathway (Pettersson and Gustafsson, 2001). Oestradiol can induce phosphorylation of Ser118, but this appears to be independent of MAP kinase (Joel *et al.*, 1998) indicating that alternative signalling pathways can act on the same residue, depending on the amount of oestradiol present in the cell.

Serine residues in the mouse ERβ, for example Ser124, are also phosphorylated by the MAP kinase pathway (Tremblay *et al.*, 1999a). In human, ERβ contains two

serine residues which are phosphorylated; Ser106, which has previously been shown to mediate Ras activation of mouse ERβ1 (Tremblay *et al.*, 1999a) and Ser106 which is part of a motif shared with other steroid receptors (Rochette-Egly, 2003).

1.12 Oestrogenic and Anti-oestrogenic Ligands

Steroid hormones are widely distributed small, lipophilic molecules that participate in intracellular communication and control of a wide spectrum of developmental and physiological processes (Beato and Klug, 2000).

Oestrogen receptors are unique in that they have the ability to bind to a wide variety of oestrogenic ligands that exhibit remarkably diverse structural features (Kuiper *et al.*, 1997). Some compounds act as receptor agonists, such as 17β-oestradiol (E_2) and certain phytoestrogens (Barkhem *et al.*, 1998; Enmark and Gustafsson, 1999; Kuiper *et al.*, 1998), whilst others function as pure antagonists such as ICI 164,384. Another category termed "selective ER modulators" (SERMs) have the ability to act as both agonists and antagonists depending upon the subtype of the receptor and the cellular and promoter context (Lonard and Smith, 2002; Paech *et al.*, 1997).

Binding studies have provided a description of the binding affinity relationships of different ligands for the receptors and a model for the ligand binding site (Anstead *et al.*, 1997). Each ligand produces distinct conformational differences, confirmed by crystallographic studies, in the receptor's LBD (McDonnell *et al.*, 1995). The oestrogenic potency of different compounds is determined by a number of factors such as the binding affinity, the induced conformational change, differential effects on the transactivation functionalities of the receptor, the particular co-activators recruited and the cell- and target gene promoter context (Kuiper *et al.*, 1998).

Table 1.2. Table to show the binding affinities of ERα and ERβ (EC_{50} nM) for a range of oestrogenic ligands.

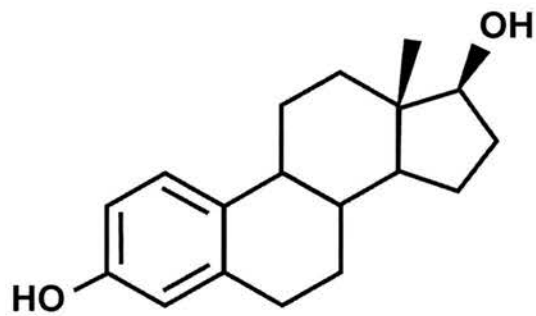
	ERα	ERβ1	Species	Reference
E_2	1.98 nM 3.2 nM	2.35 nM 3.6 nM	Human Human	Harris *et al.*, 2002a; Miller *et al.*, 2003
Genistein	334 nM 395 nM	6.65 nM 10 nM	Human Human	Kuiper *et al.*, 1997 & Harris *et al.*, 2002a; Miller *et al.*, 2003
Tamoxifen	197 nM	206 nM	Human	Harris *et al.*, 2002a
Raloxifene	7.9 nM	44.0 nM	Human	Harris *et al.*, 2002a
ICI	6.4 nM	5.8 nM	Human	Harris *et al.*, 2002a
PPT™	0.98 nM	-	Human	Sun *et al.*, 2002
DPN™	66 nM	0.85 nM	Human	Meyers *et al.*, 2001

NB. 3βAdiol EC_{50} has not been documented.

1.12.1 17β-Oestradiol

Oestradiol is the most potent oestrogen produced in the body and is considered the "classical" ligand for the oestrogen receptor. It is synthesised from testosterone or oestrone by the aromatase or 17β-hydroxysteroid dehydrogenase (HSD) enzymes. The two receptor subtypes, ERα and ERβ1 have a similar affinity for 17β-oestradiol (Enmark and Gustafsson, 1999; Harris *et al.*, 2002a) (see *Table 1.2*), despite sequence differences within their LBDs (Kuiper *et al.*, 1996; Ogawa *et al.*, 1998b). *Figure 1.18* shows the chemical structure of 17β-oestradiol. The aromatic A-ring, the B- and C-rings and the OH-group in the D-ring all contribute significantly to the receptor binding (Anstead *et al.*, 1997).

Figure 1.18. Chemical structure of 17β-oestradiol.



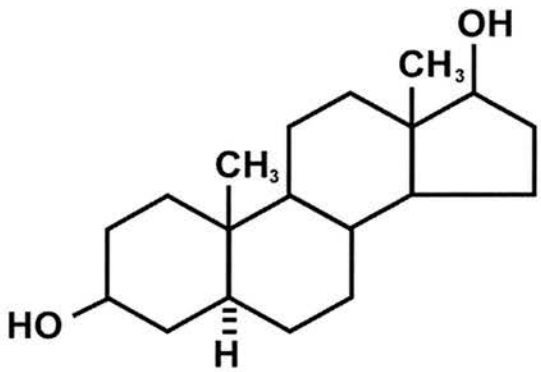
1.12.2 5α-androstane-3β,17β-diol (3βAdiol)

The potent 5α-dihydrotestosterone (DHT) androgen is converted to two metabolites in rodents; 5α-androstane-3α,17β-diol (3αAdiol) and 5α-androstane-3β,17β-diol (3βAdiol) (*Figure 1.19*) (Weihua *et al.*, 2003). Whereas in human it is thought only 3βAdiol is formed (unpublished observation by Ian Mason). Both are epimers but have very different biological actions; 3αAdiol is androgenic whereas 3βAdiol is oestrogenic (Weihua *et al.*, 2003). 3αAdiol functions as a weak androgen by itself and through the action of 3α-hydroxysteroid dehydrogenase (3βHSD) is reconverted to the potent androgen DHT (Pilven *et al.*, 1976), however it has no oestrogenic activity or effects. The oestrogenic activity of 3βAdiol has been well documented and the ligand has been shown to displace E_2 from ERα (Hackenberg *et al.*, 1993; Ho and Ofner, 1986). The hydroxylation enzyme, CYP7B1, terminates 3βAdiol's oestrogenic activity (Weihua *et al.*, 2003). Compared to E_2, 3βAdiol's affinity is 10-fold lower for ERβ and 20-fold lower for ERα (Kuiper *et al.*, 1997).

In vivo studies have been performed with mice although it has been difficult to show the oestrogenic activity of 3βAdiol (reviewed in Weihua *et al.*, 2003). In rats high doses of 3βAdiol were observed to have an effect on uterine growth and vaginal stratification, however at lower doses there were no uterotrophic effects but a delay of ovulation was recorded (Kramer and Meijs-Roelofs, 1982). These *in vivo* data had led endocrinologists to believe that 3βAdiol did not perform physiological functions by itself (Kramer and Meijs-Roelofs, 1982). However recently the regulation of

3βAdiol by CYP7B1 has been proposed to be a key factor in regulation of prostatic growth (Weihua *et al.*, 2003).

Figure 1.19. Chemical structure of 3βAdiol.



In the prepubertal female rat the serum 3βAdiol levels are high, around 100ng/ml, after the first ovulation levels are decreased to 10pg/ml (Eckstein, 1974). The origin of 3βAdiol in the immature animal is the ovary. In mature male rats serum levels of 3βAdiol are around 200pg/ml, this is about 20 times higher than the oestradiol level. Even though the binding affinity for 3βAdiol is 10-fold lower than that of E_2, 3βAdiol could still serve as a physiological ligand for ERβ. For example, it has been proposed that 3βAdiol binds to ERβ with agonistic affects and in the prostate this results in the inhibition of androgen receptor expression (Weihua *et al.*, 2002).

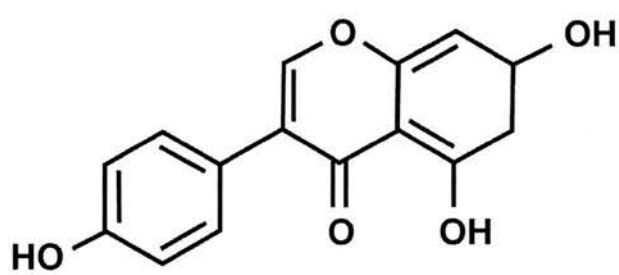
1.12.3 Genistein

Phytoestrogens are a diverse group of non-steroidal polyphenolic oestrogenic compounds produced by plants primarily as bacterial and fungicidal agents. They represent the major natural exogenous source of oestrogenic molecules (Adlercreutz and Mazur, 1997; Pettersson *et al.*, 2000). The presence of such compounds in the human diet appears to be beneficial and may even reduce the risk of certain cancers and heart disease (Adlercreutz and Mazur, 1997; and reviewed in Strauss *et al.*, 1998).

Genistein is an iso-flavonoid phytoestrogen that is found in significant levels in soya bean and soy products. It has a similar structure to oestradiol (see *Figure 1.20*). Upon binding, genistein is completely buried in the hydrophobic core of the ERα's LBD and induces conformational changes within the AF-2 region, similar to that

observed with E_2 (Pike *et al.*, 1999). There have been many studies analysing the binding affinities of genistein for ERα and ERβ1, with a number of different values being obtained (An *et al.*, 2001; Barkhem *et al.*, 1998; Harris *et al.*, 2002a) (see *Table 1.2*). Overall, genistien shows full agonism for ERα and only partial agonism for ERβ1 (Liu *et al.*, 2003; Pettersson and Gustafsson, 2001). In the ERβ-genistein complex helix 12 does not adopt the distinctive agonist position, but instead forms an intermediate position in a similar orientation to that induced by other ER antagonists (Lonard and Smith, 2002; Pike *et al.*, 1999). This was unexpected as the structures of E_2 and genistein are similar (Pike *et al.*, 1999). However, the difference in binding affinity of genistein for ERβ1 does not translate directly into the observed transcriptional activation of a 1x vitellogenin ERE reporter because genistein is over 1000-fold more potent at triggering transcriptional activity via ERβ1 compared with ERα in U937 cells (An *et al.*, 2001; Barkhem *et al.*, 1998). It has been suggested that the molecular mechanism for ERβ1 selectivity by genistein involves the receptor's capacity to create an AF-2 surface that has a greater affinity for co-activators than that formed in ERα-genistein complexes (Tremblay *et al.*, 2001).

Figure 1.20. Chemical structure of genistein.



1.12.4 Synthetic Oestrogen Receptor Modulators (SERMs)

Synthetic oestrogen receptor ligands such as tamoxifen and raloxifene produce biological responses which can be either oestrogenic or anti-oestrogenic, depending upon the tissue in which their action is examined (Lonard and Smith, 2002). These

ligands have therefore been termed selective oestrogen receptor modulators (SERMs). They are able to exert different effects through ERα and ERβ due to their differential ability to alter the ER's LBD conformation and thereby affecting the receptor's ability to bind to EREs and recruitment of co-factors (Norris *et al.*, 1999; Paige *et al.*, 1999).

Both raloxifene and tamoxifen inhibit AF-2 activity of ERα and ERβ1. Tamoxifen is thought to stimulate ERα mediated transcription through the N-terminal AF-1 in certain cellular or promoter contexts (Metzger *et al.*, 1995). Evidence from studies in Hep G2 cells suggests that this activity can be supported through the classical mechanism of ERα action, namely through the interaction of the ligand bound receptor with an ERE-containing DNA response element (Hall and McDonnell, 1999). Tamoxifen acts as a pure antagonist on ERβ1 as it inhibits transcriptional activation via AF-2 and the AF-1 domain in ERβ1 is reported to possess negligible transcriptional activity (Brzozowski *et al.*, 1997; Hall and McDonnell, 1999).

It is uncertain whether raloxifene can stimulate gene transcription through the AF-2 or AF-1 domains in the classical pathway of ER dependent gene expression (McDonnell *et al.*, 1995).

1.12.5 Specific ERα or ERβ1 Synthetic Ligands

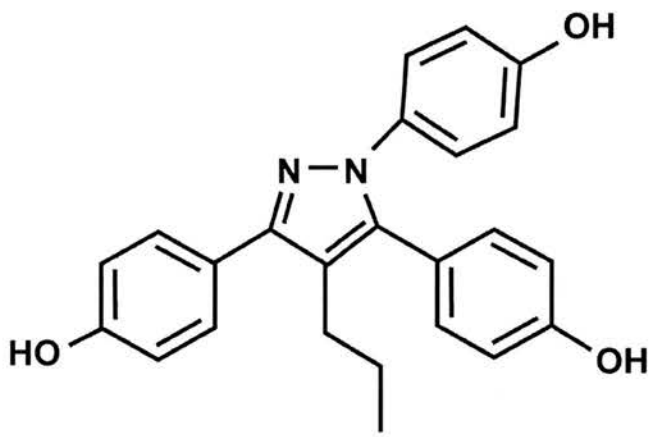
ERα and ERβ only share 56% amino acid homology within their LBDs and although the residues that surround the ligand are nearly identical ER subtype selective ligands have been developed (Meyers *et al.*, 2001; Sun *et al.*, 2003; Sun *et al.*, 2002). Such subtype selective ligands could prove to be useful tools in probing the physiological functions of each ER subtype.

1.12.5.1 PPT™

Propyl pyrazole triol (PPT™) is a nonsteroidal ligand, with the highest affinity for ERα of all the pyrazole compounds (Stauffer *et al.*, 2000) (*Figure 1.21*). PPT™ is a potent agonist on ERα, with a high relative binding affinity (49%+/-12% that of E_2) but is reported to be inactive when incubated with ERβ, having a very low binding affinity (0.12%+/-0.4% that of E_2) (see *Table 1.2*) (Kraichely *et al.*, 2000). PPT™ stabilizes ERα to the same extent as E_2 upon binding and is believed to induce a similar agonist-like conformation, recruit co-activators such as SRC-1 and therefore

initiates gene transcription to a similar extent to E_2 (Kraichely *et al.*, 2000). However as PPT™ is reported not to be an agonist for ERβ1 as it fails to induce recruitment of co-activators and does not stimulate gene transcription (Kraichely *et al.*, 2000).

Figure 1.21. Chemical structure of PPT™.

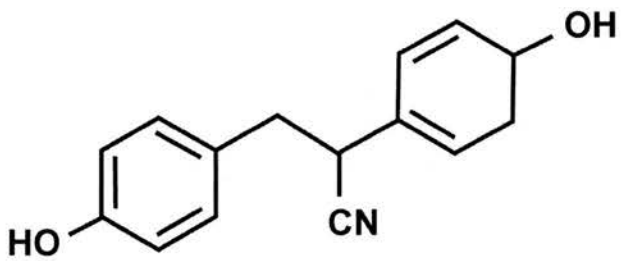


1.12.5.2 DPN™

Meyers *et al* recently prepared a non-steroidal ERβ1 potency-selective ligand; 2,3-bis(4-hydroxyphenyl)propionitrile (DPN™) (Meyers *et al.*, 2001) (*Figure 1.22*). DPN™ acts as an agonist on both ERα and ERβ1 subtypes, but has a 70-fold higher relative binding affinity (see *Table 1.1*) and a 170-fold relative potency in transcription assays with ERβ1 compared to ERα (Meyers *et al.*, 2001). This is a substantially higher level of ERβ1 affinity and potency selectivity than observed with the partial agonist genistein (Meyers *et al.*, 2001).

Site-specific mutagenesis and DNA shuffling has demonstrated that one relatively conserved residue in the LBD between hERα and hERβ1 (L384 in ERα and M336 in ERβ1) is important in DPN™ binding and that the hERβ1 selectivity for DPN™ can be significantly altered (Sun *et al.*, 2003). This one amino acid change can affect not only the local interaction with ligand, but it also has a long-range influence on other parts of the binding pocket (Sun *et al.*, 2003). However other residues at the beginning of helix 3 of the LBD are also very important. Hence a limited number of critical interactions of DPN™ with the ERβ1 LBD underlie its ER subtype selective character (Sun *et al.*, 2003).

Figure 1.22. Chemical structure of DPN™.



1.13 Tissue Specific Patterns of Expression of Oestrogen Receptors

As shown in *Figure 1.1* oestrogens have an effect on a wide range of tissues, and consistent with this, oestrogen receptors are widely distributed throughout the body.

1.13.1 Expression of ERα and ERβ

The ovaries are the main source of oestrogen in non-pregnant premenopausal women, consistent with the expression of high levels of cytochrome P450 in the granulosa cells of the preovulatory follicles (Simpson and Davis, 2001). Oestrogens are essential for normal follicular maturation in the human as well as in the primate (Palter *et al.*, 2001). Messenger RNAs for both ERα and ERβ have been detected in ovarian extracts from a wide range of species including rodents (Byers *et al.*, 1997), primates and humans (Misao *et al.*, 1999). ERα and ERβ mRNAs have been reported to be differentially expressed within the ovary. For example in rodents ERβ mRNA is predominantly expressed within granulosa cells (Byers *et al.*, 1997) whereas ERα mRNA has been detected in human corpus luteum (Brandenberger *et al.*, 1998). Overall, total ovarian mRNA for ERβ is more abundant than for ERα (Drummond *et al.*, 1999; Saunders *et al.*, 2000).

In the rodent ovary immunohistochemical analysis has detected ERα protein in stromal nuclei, germinal epithelium and theca but not in granulosa cells (Sar and Welsch, 1999; Saunders *et al.*, 1997; Suzuki *et al.*, 1994). Saunders *et al* were the first to immunolocalise ERβ protein to rodent granulosa cells of all stages of follicular maturation (Saunders *et al.*, 1997). Others have confirmed and extended these

results (Fitzpatrick *et al.*, 1999; Sar and Welsch, 1999). In humans and primates ERα has been immunolocalised to granulosa cells of mature antral follicles (Saunders *et al.*, 2000). ERβ protein has been detected in granulosa cells and cells within the corpus luteum (Saunders *et al.*, 2000). ERα and ERβ proteins have also been localised to the surface epithelium of the ovaries (Henderson *et al.*, 2003; Saunders *et al.*, 2000). This is consistent with detection of mRNAs for ERα and ERβ in cultured ovarian epithelial cells (Hillier *et al.*, 1998).

The uterus is a target for oestrogen action and all the cellular components of the endometrium (the epithelial, stroma, endothelium and bone-marrow derived cells in the stroma) express one or both of the oestrogen receptors (Saunders, 2002). The cyclical event of menstruation follows the sequential exposure of the endometrium to oestrogen and progesterone. Messenger RNAs for both ERα and ERβ have been detected within glandular epithelial cells, stromal cells and the smooth muscle of the uterine wall at every stage of the menstrual cycle (Critchley *et al.*, 2002). During the proliferative phase ERα mRNA may be approximately 300-fold higher than ERβ mRNA (Matsuzaki *et al.*, 1999). Henderson *et al* confirmed that mRNAs for ERβ1 and the ERβ2 variant were both present in human endometrial extracts across the menstrual cycle, increasing during the late secretory phase (Henderson *et al.*, 2003).

Immunohistochemistry has localised ERα to glandular and stromal cell nuclei in the endometrium and a decline in immuno-intensity of ERα has been reported in the superficial layer in the secretory phase (Henderson *et al.*, 2003; Lecce *et al.*, 2001; Saunders, 2002). In contrast, ERβ protein expression does not decline during the secretory phase (Critchley *et al.*, 2001; Lecce *et al.*, 2001). There was a difference in ERα and ERβ immuno-expression in the vascular endothelium and the perivascular cells surrounding the endometrial blood vessels. Only ERβ protein was detected in the endothelial cell population, although both ERα and ERβ were found in perivascular cells (Critchley *et al.*, 2001; Critchley *et al.*, 2002; Lecce *et al.*, 2001; Saunders, 2002).

Human decidual cells from early pregnancy contain positive nuclear immunostaining for ERα protein (Wu *et al.*, 1993); ERβ has been detected in the decidualised stromal cells (Lecce *et al.*, 2001). However, in the third trimester ERα protein has

not been detected in decidual tissue (Noci *et al.*, 1994). In agreement with this Saunders *et al* reported expression of ERβ but not ERα in the nuclei of cells of the syncytiotrophoblast of the placenta during the second trimester and at term (Saunders, 2002).

It has been proposed that oestrogens play a role in regulating spermatogenesis (reviewed by O'Donnell *et al.*, 2001). Levels of oestrogens within the male reproductive tract are higher than in the general circulation (Hess *et al.*, 1997). The distribution of ERα and ERβ mRNAs within the male reproductive tract are tissue and cell specific (Enmark *et al.*, 1997; Williams *et al.*, 2001). Messenger RNA for ERβ has been found in developing spermatids of human testis, suggesting that oestrogens participate in the regulation of spermatogenesis (Inoue *et al.*, 2000), however ERα is absent (Scobie *et al.*, 2002). In the rodent some studies have claimed that ERα is present in Leydig cells and some peritubular cells (Zhou *et al.*, 2002). Whereas Saunders *et al* reported that the expression of ERα was confined to rodent Leydig cells (Atanassova *et al.*, 2000; Fisher *et al.*, 1997; Saunders *et al.*, 1998).

In the human and primates some studies report that ERα is not expressed in the testis (Makinen *et al.*, 2001; Saunders *et al.*, 2001), although others have reported detection of protein using squash preparations (Pentikainen *et al.*, 2000). High levels of expression occur in the efferent ductules and in the initial segment of the epididymis that lie adjacent to the testis (Saunders *et al.*, 2001). The detection of ERα mRNA in human testis extracts may occur if the tissue is not dissected carefully with the efferent ducts separated from the testis (Saunders *et al.*, 2001).

1.14 Mouse Models of Receptor Knockouts

Recently four mouse strains have been created in which either ERα or ERβ or both receptors, or the hormone; oestradiol, have been eliminated (Enmark and Gustafsson, 1999). The two oestrogen receptors were inactivated through targeted disruption of the respective ER genes, and oestradiol was removed by inactivation of the aromatase enzyme (*cyp19*) which converts testosterone to oestradiol (Fisher *et al.*, 1998).

1.14.1 αERKO Mice

Female αERKO mice become infertile by four or five weeks of age (Lubahn *et al.*, 1993). They initially exhibit normal follicular development, but in adulthood the follicles tended to become atretic after the antral stage with no apparent corpora lutea, finally resulting in haemorrhagic cysts. The development of these cysts appears in part to be due to elevated levels of gonadotrophins (LH and FSH) caused by a loss of negative feedback at the level of the pituitary (Schomberg *et al.*, 1999). In agreement with this, prevention, using a GnRH antagonist inhibits development of these cystic structures (Couse and Korach, 1999). The uteri of αERKO female mice are immature in appearance and lack development in adulthood (Lubahn *et al.*, 1993).

Male αERKO mice undergo apparently normal prenatal development to produce internal and external structures that are indistinguishable from wild type males. However as adults there is a significant reduction in testis weight compared to wild type litter mates (Couse and Korach, 1999). Within the testis hyperplastic Leydig cells are observed, consistent with increased LH levels. The adults are infertile, with a significantly reduced sperm count and decreased sperm mobility (Couse and Korach, 1999). Their rete testis and efferent ductules are dilated (Hess *et al.*, 2000). Detailed investigations suggest that infertility in αERKO males is a result of reduced fluid resorption within the efferent ductules. This leads to an increased back pressure and collapse of the seminiferous tubule resulting in the loss of germ cells (Hess *et al.*, 2000).

The sperm from αERKO males have a lower percentage mobility, beat less vigorously and show less forward progression than sperm from wild type mice (Lubahn *et al.*, 1993; Mahato *et al.*, 2000). Mahato *et al* suggested that ERα does not regulate genes in the germ cells, required for their development in the testis or maturation in the male duct system, but rather affects sperm function indirectly through somatic cells that support spermatogenesis and/or epididymal function.

1.14.2 βERKO Mice

Female βERKO mice contain all stages of follicular development, however as adults ovulation rate is reduced (Krege *et al.*, 1998). In addition, litter size is reduced and substantially fewer litters are produced (Krege *et al.*, 1998). Histological analysis of

βERKO ovaries showed an increase in number of early atretic follicles and few corpora lutea (Couse *et al.*, 1999). This is consistent with a role for ERβ in the granulosa cells (Dupont *et al.*, 2000). The uteri of βERKO female mice appear to be comparable to those of wild type animals and although they exhibit subfertility, they are able to carry pregnancies successfully to full term (Krege *et al.*, 1998).

Male βERKO mice are fertile (Couse and Korach, 1999). The lack of a phenotype in βERKO mice appears at odds with the observation that germ cell loss occurs in male ARKO mice appearing to be consistent with oestrogen action via ERβ, expressed in spermatocytes and spermatids (de Ronde *et al.*, 2003).

1.14.3 αβERKO Mice

The ovaries of αβERKO mice are distinctly different from the single knockouts with some abnormal follicles, no corpora lutea and the presence of large cells with abundant cytoplasm and a prominent nucleus (Dupont *et al.*, 2000). Follicles with a normal morphology decreased with age as those with an unusual appearance increased. The abnormal follicles lacked an oocyte and contained cells which shared morphological characteristics with Sertoli cells. For example, there was evidence of the formation of unique junctional complexes as well as the synthesis of anti-Mullerian hormone and Sox9 mRNAs (Couse *et al.*, 1999). It has been suggested that follicles are initially normal but begin to "trans-differentiate" into seminiferous tubule-like follicles as the oocyte degenerates. The uteri of αβERKO mice do not exhibit sensitivity to oestrogens and normal growth is prevented (Couse *et al.*, 1999).

Male αβERKO mice are infertile (Dupont *et al.*, 2000). They exhibit a loss of germ cells in the seminiferous tubules and a marked dilation of straight tubules and the rete testis.

1.14.4 ARKO Mice

Female ARKO mice become infertile and display disturbances in ovarian development with follicular arrest, hyperplastic stroma and the formation of haemorrhagic cysts by one year of age (Britt *et al.*, 2000; Fisher *et al.*, 1998). The ovaries contained numerous follicles with abundant granulosa cells and evidence of antrum formation that appeared to be arrested before ovulation.

Male ARKO mice display more or less normal fertility when young, but a decrease in litter siring is apparent with advancing age (Fisher *et al.*, 1998). Adult onset of infertility observed in male ARKO mice is consistent with a role for oestrogens in the male as well as in the female (Robertson *et al.*, 1999). Analysis of the testes from the ARKO mice has revealed that round spermatids undergo apoptosis, display disturbances in acrosome formation and fail to differentiate into mature elongate spermatids (Robertson *et al.*, 2001). These results suggest oestrogens are essential for normal spermatogenesis and for a direct impact of oestrogens on male germ cell development and hence fertility.

Characterisation of the phenotypes exhibited by these knockout models has extended our understanding of the physiological processes governed by oestrogens and their phenotypes are summarised in *Table 1.3*.

Table 1.3. Details of the knockout mice phenotypes.

	E_2 levels	LH levels	FSH levels	Fertility	References
ARKO	Decreased	Decreased	Increased	Infertile females Infertile males	Fisher *et al.*, 1998
αERKO	Increased	Increased	Increased	Infertile females Subfertile males	Couse and Korach, 1999
βERKO	Increased	Normal	Normal	Subfertile females Fertile males	Hewitt and Korach, 2003
αβERKO	Increased	Increased	Increased	Infertile females Infertile males	Couse *et al.*, 1999; Dupont *et al.*, 2000

Data to support the importance of oestrogen mediated gene expression within the female reproductive tract also comes from mice that have had the co-activators SRC-1 or SRC-3 disrupted. In these mice there is partial hormone resistance and reduced female reproductive function (Wu *et al.*, 2002; Xu *et al.*, 1998).

1.15 ERE-Reporter Mice

As an alternative technique to analyse oestrogen's action *in vivo*, Ciana *et al* engineered the mouse genome to express an ERE linked to a luciferase reporter gene ubiquitously (Ciana *et al.*, 2001). They showed that oestrogen action via the ERE reporter was localised to a number of different tissues including the

reproductive tract and bone (Ciana *et al.*, 2001). Nagel *et al* also developed a transgenic mouse that functions as a reporter of ER activity. This model was used to identify target tissues that contain active ERs and thus aid in the evaluation of the contributions of the cellular environment, rather than promoter context (Nagel *et al.*, 2001). They incorporated the 3x Vitellogenin ERE linked to a minimal TK promoter with a beta-galactosidase reporter, and termed it an ER action indicator (ERIN) model. Evaluation of ER activity in the ERIN female demonstrated oestrogen-inducible expression of the reporter gene in the uterus, pituitary and hypothalamus as well as in non-classical oestrogen target tissues such as the kidney, liver, adrenal and thyroid gland. However there are other potentially important ER-mediated actions that function through pathways besides direct binding to ERE, for example, interactions with AP-1 (Paech *et al.*, 1997) to consider.

1.16 Human Cases

The roles played by oestrogens in normal human testes function are less clear than animal models due to the difficulty in obtaining tissue samples. There has been one case reported of a young male patient with an inactive ERα, who had apparently normal testes, normal sperm density but decreased sperm viability (Smith *et al.*, 1994).

There are currently four men world wide who are characterised with aromatase deficiency (taken from Simpson 2003, Abstract 85th Meeting Endo Soc. Philadelphia p40). These individuals have a range of phenotypes including tall stature due to failure of epiphysial closure, delayed bone age and a "Metabolic Syndrome". The testicular phenotype is confusing due to markedly variable penetrance and ranges from moderate oligospermia to cryptorchidism.

To date, no patients lacking expression of the ERβ gene have been identified.

1.17 Conclusions

Oestrogens have a key role in regulation of many biological processes including reproduction. The "simple" oestrogen receptor pathway has become extremely complex as our understanding of receptor isoforms and splice variants, receptor localisation, different ligands, co-factor proteins, classical EREs and non-classical alternative pathways and the activation of the receptor via a MAPK phosphorylation pathway have become apparent.

1.18 Aims

The aims of this project are; i) to determine whether ERβ wild type (ERβ1) and ERβ variant (ERβ2) forms exist in primates as well as human and to determine the ER localisation in reproductive tissues (Chapter 3); ii) to examine the localisation or absence of fluorescently tagged ER constructs (ERα, ERβ1 and ERβ2) in live cells in the presence or absence of different ligands (Chapter 4); iii) to examine the functional dose response of ER proteins to different ligands in the presence of ERE-luciferase reporter constructs (Chapter 5); iv) to investigate an alternative, non-classical activation mechanism for ERβ2 via a growth factor signalling pathway (Chapter 6).

Chapter 2

General Materials and Methods

2.1 Cell Lines

Two different cell lines were used; Hek 293 and Hep G2. Both were purchased from European Collection of Cell Culture (ECACC). The Hek 293 cells were derived from human embryonic kidney cells, they were previously transformed with sheared human Ad5 DNA. The Hep G2 cell line was isolated from a liver biopsy of a male Caucasian aged 15 years, with a well differentiated hepatocellular carcinoma. Cos 7 (African Green Monkey), Ishikawa (human endometrium), LnCap (human prostate) and Hela (human ovarian cancer) cells were obtained from stocks at the MRC Human Reproductive Sciences Unit.

2.2 Ligands

Different ligands were selected to stimulate the transiently transfected cells.

2.2.1. Natural Oestrogenic Ligands

The natural oestrogen; 5 alpha-androstane-3-beta,17 beta-diol (3βAdiol) is a metabolite of dihydrotestosterone. It was purchased from Sigma (Catalogue Number; A-7630). 17β-Oestradiol (E_2) was purchased from Sigma (Catalogue Number; E-8875). Both were reconstituted in 100% ethanol, serial dilutions (10^{-3}M to 10^{-11}M) made in 100% ethanol and stored at -20°C. 4',5,7-Trihydroxyisoflavone (Genistein) was a synthetic powder purchased from Sigma (Catalogue Number; G-6649). The powder was reconstituted in DMSO, serial dilutions produced (10^{-3}M to 10^{-11}M) with DMSO and stored at -20°C.

2.2.2 Receptor Selective Synthetic Ligands

DPN™ and PPT™ were purchased from Tocris Cookson Ltd. 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN™) is marketed as a highly potent ERβ1 agonist with a 70-fold selectivity over ERα (EC_{50} values are 0.85 and 66nM repectively) (Meyers *et al.*, 2001). The ligand 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT™) is marketed as a highly potent ERα agonist which displays a 410-fold selectivity for ERα over ERβ (Kraichely *et al.*, 2000; Stauffer *et al.*, 2000). Both of

the compounds were reconstituted in 100% ethanol and serial dilutions produced (10^{-3}M to 10^{-11}M) with 100% ethanol and stored at -20°C.

2.2.3 Growth Factors

Epidermal Growth Factor (EGF) fragment was purchased from Sigma (Catalogue Number; E9384). The lyophilised powder was reconstituted in PBS and serial dilutions made in PBS (10^{-3}M to 10^{-11}M). Aliquots were stored at -20°C. Insulin-like Growth Factor-1 (IGF-1) was purchased from Sigma (Catalogue Number; I-3769). The IGF-1 was a human recombinant single chain polypeptide of 70 amino acid residues cross-linked by three disulphide bridges, expressed in *E.coli*. The lyophilised powder was reconstituted in 10mM HCl to a concentration of 2.0mg/ml, serial dilutions made (10^{-3}M to 10^{-11}M) in 100% ethanol and stored at -20°C.

2.3 RNA Extraction

A typical mammalian cell contains about 10^{-5}µg RNA, 1-5% is mRNA, which is heterogeneous in both size and sequence. The other 80-85% is rRNA and 15-20% is low molecular weight tRNAs, nRNAs etc. (Sambrook *et al.*, 1989). The mRNA was extracted from cells or tissue samples using the commercial Tri Reagent purchased from Sigma (Chomczynski, 1993).

2.3.1 RNA Extraction from Cells

A cell suspension was prepared from a single confluent 75cm^2 flask by adding 2mls 1x trypsin (Sigma, Catalogue Number; T-4424) and incubating at room temperature until the cells detached from the flask. The trypsin was neutralised by the addition of fresh media (section 2.10.1), the cells were counted using a haemocytometer and centrifuged at 1000 rpm for 5 minutes at room temperature to pellet. The supernatant was discarded and RNA was extracted from the pellet using Tri Reagent (Sigma) according to manufacturers instructions. In brief, 1ml Tri Reagent was added to the cell pellet on ice and thoroughly mixed by pipetting, followed by the addition of 200µl chloroform, the sample was mixed on a vortex and left to stand at room temperature for 5 minutes. The aqueous and organic layers were separated by centrifugation at 12,000 rpm for 10 minutes at 4°C. The aqueous layer (approximately 600µl) containing the RNA was transferred to a fresh chilled Eppendorf tube (1.5ml) which was placed on ice and 500µl isopropanol (pre-chilled to –20°C) added to induce the precipitation of RNA. The sample was mixed gently

by inverting the tube and left to stand for 10 minutes on ice. The sample was centrifuged again at 12,000 rpm at 4°C for 10 minutes, the supernatant removed and the RNA pellet washed with 75% ethanol, air-dried and re-suspended in RNA storage solution (Ambion). The concentration and purity of the RNA was measured using a Pharmacia Biotech Genequant (section 2.6.2). The sample was aliquoted and stored at -70°C.

2.3.2 Extraction of RNA from Tissues

Frozen tissue samples were ground to a fine powder under liquid nitrogen using a pestle and mortar. The sample was divided up into approximately 50mg aliquots and stored at -70°C. For RNA extraction approximately 50-100mg tissue was required. Extraction of RNA was performed using Tri Reagent as in 2.3.1 and the RNA re-suspended in RNA storage solution.

2.4 Preparation of Complementary DNA (cDNA) by Reverse Transcription (RT)

The cDNA was synthesised from total RNA using oligo dT primers (Invitrogen) and pools of each dNTP (Invitrogen). An aliquot of total RNA (5μg) prepared using the methods in section 2.3, was mixed with 2μl RNasin (Promega) and the volume adjusted to 21μl using RNAse-free water (Promega). Oligo dT (2μl, 200pM) primers were added to the reaction and the sample incubated at 75°C for 3 minutes to reduce secondary structure within the RNA. Thereafter the reaction was placed on ice to encourage annealing of the oligo DT primer to the poly A+tail at the 3' end of mRNAs. In a separate tube 4μl 100mM DTT (Roche), 8μl 5x RT buffer (50mM Tris-HCl, 100mM NaCl, 1mM EDTA, 10mM DDT, 0.05% polydocanol (v/v), 50% glycerol (v/v); pH 8.4), 4μl 10mM total dNTPs (dATP, dCTP, dGTP and dTTP) and 1μl DNase (Sigma) were mixed together. 2μl Expand RT^{TM} was added to the master mix (19μl final volume) which was then added to the RNA sample to give a final reaction volume of 40μl. A control was included using pure water (Promega) to replace the RNA. The RT reaction was incubated at 42°C for 2 hours, thereafter the reaction was terminated by heating at 99°C for 5 minutes. A volume of 1μl RNase was added to the single stranded cDNA and incubated at 37°C for 15 minutes to remove RNA. The cDNAs were separated from unincorporated nucleotides and primers using "High Pure Purification columns" (Roche) following manufacturers

guidelines (section 2.7.2) and analysed using a Pharmacia Biotech Genequant (section 2.6.2).

The quality of the cDNA pool was checked by using PCR (section 2.5) with primers specific for the housekeeping gene GAPDH. PCR products were separated on a 1% agarose gel stained with ethidium bromide and photographed (section 2.6.1).

2.5 Amplification of Specific cDNAs by Polymerase Chain Reaction (PCR)

The single stranded cDNA pools synthesised from section 2.4 were used in PCR reactions with oligonucleotide primers specific to target sequences to determine whether specific RNA messages were present within the cell or tissue used for RNA extraction.

The PCR reactions are dependent upon the synthesis of DNA by a thermostable DNA polymerase (Saiki *et al.*, 1988). The *Thermus aquaticus* (Taq) enzyme can survive extended incubation at 95°C and therefore is not inactivated by the heat denaturation step in each cycle. The buffer used in the reaction contains 50mM KCl, 10mM Tris-HCl; pH 8.3, and 1.5mM $MgCl_2$. During the 72°C amplification step the pH decreased by more than a full unit producing a buffer with a pH of approximately 7.2. The presence of divalent cations was essential for the reaction, Mg^{++} concentration was fairly low (1.5mM) therefore it was necessary to ensure that the concentration of chelating agents such as EDTA were kept minimal. The dNTPs were used at saturating concentrations (200μm for each dNTP) to ensure they didn't become depleted and the efficacy of the reaction was not reduced after multiple cycles.

2.5.1 Oligonucleotide Primers

Oligonucleotide primers (17-24mer) were synthesised by Sigma-Genosys and MWG Biotech. The primers were suspended in either TE (10mM Tris-HCl; pH 7.5, 1mM EDTA) or pure water (Promega) to obtain a concentration of 50pmol/μl.

The primers were chosen to contain approximately 50% G+C content and an annealing temperature within the range of 55-62°C. In the current studies primers were chosen to sequences within the open reading frame of the mRNAs but in the case of large gene families such as the steroid receptors regions with significant sequence homology between family members e.g. the DNA binding domain, were

avoided. The length of the primer and its GC content determines both the specificity and the annealing temperature used during the PCR reaction. A primer of 17-24 nucleotide bases is sequence specific as long as the annealing temperature is set within a few degrees of the dissociation temperature for the template/primer duplicate. Using primers of a minimal length (17 bases) ensured a melting temperature of around 54°C or higher and a high probability of sequence specificity.

Table 2.1. Screening primers used to detect cDNAs in pools prepared from cells or tissues.

	5' primer	3' primer	Product size
ERβ1	GGCATCTCCTCCCAGCAGCA	CACTGAGACTGTGGGTTCTGGA	240 bp
ERβ2	GGCATCTCCTCCCAGCAGCA	CACTGCTCCATCGTTGCTTC	135 bp
EGFr	GTGTGCCCACTACATTGACGC	GTTGGACAGCCTTCAAGACC	237 bp
IGFr	ATGCTGTTTGAACTGATGCG	TCTCCATGTTCTCTGGCTCC	166 bp
SRC-1	CATGCTTATGAGGCAGCAAA	ATTCCAGTGCCAAACTGTCC	247 bp

Table 2.2. Primers used to amplify full length cDNAs.

	5' primer	3' primer	Product size
hERβ1	CCACGAATCTTTGAGAACATTAT	GTGGGCCAGTTCACCTCAGG	1698 bp
hERβ2	CCACGAATCTTTGAGAACATTAT	GTGAAAATGTTACCCAAGAT	1615 bp

ATG start sites are approximately 66 bp downstream from the 5' primers

Table 2.3. Primers used for cloning using ECHO Vectors.

	5' primer	3' primer	Product
H ERβ1	GACATGGATATAAAAAACTCACC	CACTGAGACTGTGGGTTCTGGGA	1591 bp
H ERβ2	GACATGGATATAAAAAACTCACC	CACTGCTCCATCGTTGCTTC	1489 bp
Mm ERβ1	GACATGGATATAAAAAACTCACC	CACGGAGACTGTGGGTTCTGGGA	1590 bp
Mm ERβ2	GACATGGATATAAAAAACTCACC	CAATCTCCATCTTCCATTCCAAA	1458 bp
Mc ERβ1	GACATGGATATAAAAAACTCACC	CACTGAGACTGTGGGTTCTGGGA	1590 bp
Mc ERβ2	GACATGGATATAAAAAACTCACC	GCGAAGGTCACAGATCCATCCGT	960 bp

ATG Start Sites

Table 2.4. ERβ primers used for sequencing.

	Primer
100 Anti Sense (3')	GCTATAGAATGTCATGGCTGGA
1760 Sense (5')	GGCATCTCCTCCCAGCAGCA
CMV (5')	CGCAAATGGGTAGGCGTG
BGH (FP) (5')	TAGAAGGCACAGTCGAGG

Table 2.5. Control primers to determine PCR quality.

	5' primer	3' primer	Product size
GAPDH	CTGCACCACCAACTGCTTAGC	ATGCCAGTGAGCTTCCCGTTC	280 bp

Table 2.6. Primers used for cloning into the FP constructs.

ERβ1	5' CGAGCTCAAGCTTTGGACATGGATATAAAAAACTCA 3' GCGCGGATCCTCACTGAGACTGTGGGTT
ERβ2	5' CGAGCTCAAGCTTTGGACATGGATATAAAAAACTCA 3' GFPGCGCGGATCCTCACTGCTCCATCGTTGC

ATG Start Site, HindIII Restriction Site, BamHI Restriction Site

Table 2.7. Sequences used to generate the vitellogenin ERE

Vit ERE	Sequence
5' primer	GTCAGGTCACAGTGACCTGATCAAAGTTAATGTAACCTCAG
3' primer	CTAGCTGAGGTTACATTAACTTTGATCAGGTCACTGTGACCTGACGTAC

Important ERE region, Nhe I restriction site, Kpn I restriction site

2.5.2 PCR Reactions

Two different sets of reagents for PCR reactions were used at different times during these studies; ABgene Extensor High-Fidelity PCR Master Mix (Advanced Biotechnologies) and Mega Mix (Microzone Ltd).

2.5.2.1 ABgene Extensor High-Fidelity PCR Master Mix

Using the ABgene Extensor High-Fidelity PCR Master Mix; 5μl 10x reaction buffer (22.5mM $MgCl_2$), 1μl Extensor Hi-Fidelity PCR Enzyme mix (Taq included) and 19μl pure water (Promega) were mixed together (total volume 25μl). Another mix was made up containing 5μl 50mM dNTP mix, 5-10ng cDNA, 100pM of each primer and made up to 25μl with pure water. Both these master mixes were added together (total volume 50μl) in a 0.2μl thin walled tube (ABgene) on ice.

2.5.2.2 Mega Mix Containing Reaction Mix

Using Mega Mix (consisting of Taq polymerase, dNTPs, 2.5mM $MgCl_2$, buffer, and enzyme stabiliser) 50pM of each primer and 2-5ng cDNA were mixed to a total volume of 10μl with the Mega Mix on ice in a 0.2μl thin walled tube.

2.5.3 PCR Cycle

For a typical PCR the reactions were subjected to an initial denaturing incubation at 95°C in a PTC-200 Thermal Cycler (MJ Research) for 2 minutes followed by 35 cycles of: 94°C for 30 seconds, annealing of primers with the thermostable DNA Taq polymerase at 60°C, 58°C or 55°C for 30 seconds depending upon the primer used, 72°C for 2 minutes, and a final 10 minute incubation at 72°C. The PCR machine had a hot lid so it was not necessary to add mineral oil to prevent evaporation. The machine was programmed accordingly with the volume of the PCR reaction. A negative control was always included, using pure water (Promega) instead of the cDNA.

2.6 Product Analysis

To ensure the product of a PCR, cloning or purification reaction was correct aliquots (5-10μl) of each sample were either visualised on a gel or the purity and concentration determined using a Pharmacia Biotech Genequant.

2.6.1 Agarose Gel Analysis

Agarose gels were used to check the size and purity of PCR products, plasmids and to determine whether ligations had worked. Size and conformation of the plasmid construct influences the migration of the DNA during electrophoresis, superhelical DNA migrates the slowest, whereas linear DNA migrates faster.

Agarose gels were prepared using 1xTAE buffer (2M Tris acetate, 50mM EDTA pH 8.0) with the addition of 1% (w/v) agarose (Roche). The percentage of agarose used was increased to 2% when the expected size of the DNA to be visualised was smaller than 300bp. The agarose was melted in a microwave oven and 1:2000 dilution of ethidium bromide solution (stock solution at 1mg/ml, Sigma) added to allow UV visualisation of the product bands. The gel was poured into a gel tray and a comb used to form the wells for the samples.

PCR products (5μl) to be run on the gel were mixed with 1μl 5x blue loading dye (0.25% bromophenol blue and 30% glycerol). When cloning products were run on a gel, an approximate 100ng concentration was loaded in a maximum volume of 15μl with the dye. The blue dye ran at approximately 600bp, therefore if the product to be run was the same size as the blue dye it was mixed with 2x Orange G (50% 1xTAE,

20% glycerol, and 0.25% Orange G; Sigma, Catalogue Number; 3756) loading dye instead which runs at approximately 50bp. Size markers were run in one lane of all gels. For PCR products (100-800bp), 5μl of a 100bp DNA ladder (Promega) was mixed with 1μl blue dye or 5μl orange dye. For larger products (plasmids/full length cDNAs) 2μl Lambda marker cut with HindIII (Promega) was used. Once the samples were loaded into the gel wells, submerged in 1xTAE in the electrophoresis tank (Bio-Rad Equipment), the gel was run at 100 volts for 30-60 minutes depending upon the product migration and gel size. The gel was visualised using a UV transilluminator (GRI Syngene) and photographed with a BioRad camera and integration control unit. When PCR products were analysed a band of a certain size, depending upon the primers used, was visible. Pure plasmid DNA appeared as two bands, one for circular DNA and the other representing supercoiled DNA. Other cloning products were analysed by comparing their size to known markers or controls. The approximate concentration of a sample was also determined by running a plasmid sample of known concentration i.e. 200ng, on the gel to compare intensity of the sample band.

2.6.2 Genequant Analysis

The Pharmacia Biotech Genequant spectrophotometer was used to determine the concentration and purity of the sample RNA, double or single stranded DNA. The blank reference was set first, using either pure water (Promega) or TE depending on the sample content and the Genequant was programmed to analyse RNA, dsDNA or sDNA samples, with an OD set at 260nm. A small volume (7-10μl) of the sample was pipetted into the cuvette and the product's concentration, ratio and absorbance reading noted. A ratio reading of 1.9 was taken to be pure; a higher ratio indicated the sample was degraded, a lower ratio indicated there was protein present in the sample. The absorbance reading should be approximately 10-fold more than the concentration reading.

2.6.3 Sequencing

All sequencing was performed using an ABI 373 or the newer ABI 377 Sequencer. Samples were run by the Sequencing Service within the MRC Human Reproductive Sciences Unit. Analysis and alignments were performed using GeneJockey II Sequence Processor (P. Taylor BIOSOFT).

2.7 Subcloning of cDNA Constructs

Purified PCR products were cloned into plasmid vectors so that they could be expressed in bacterial cells, or introduced into eukaryotic cells by transient transfections, for Luciferase assays and protein expression studies.

2.7.1 Amplification and Cloning of Full Length ERβ cDNAs

Primers 5' of the human ATG start and 3' of the human TGA stop were chosen and used to amplify full length ERβ1 and ERβ2 cDNAs from human, macaque and marmoset testes using the PCR reagents detailed in section 2.5. However, a reduced primer annealing temperature and an increased extension time of 1 minute 30 seconds was used to amplify up the full length products. PCR products were purified (section 2.7.2) and sequenced using the original PCR amplification primers and a series of internal sequencing primers generated to human ERβ sequence (*Table 2.4* and section 2.6.3) to ensure the sequence of the PCR product was correct and that no base changes had been introduced during the PCR reaction.

Human ERα was purchased from the American Type Culture Collection (Catalogue Number; 79762). The hERα cDNA was restriction digested out of the pIC2OH vector it was supplied in, using BamHI and EcoRV restriction sites located in the vector's multiple cloning site. In a total volume of 50μl, 5μg ERα-pIC2OH, 2.5μl of each enzyme (Promega) and 5μl Buffer (Promega) was made up with water and incubated for approximately 4 hours at 37°C to digest. The reaction mix was purified by eluting it through a High Pure Purification column (section 2.7.2).

2.7.2 Purifiying cDNA and PCR Products Prior to Cloning

The cDNAs prepared as in section 2.7.1 and restriction digested products (section 2.8.2) used for cloning and ligations were purified to remove the Taq and other contaminants which have a high salt content and may have inhibited cloning reactions. In order to achieve this the product was passed through either a Chromo-spin TE-400 column (Clontech) or a High Pure Purification column (Roche) according to the manufacturer's instructions. Using the Chromo-spin TE-400 column, the column was centrifuged at 1800 rpm for 3 minutes and the flow through discarded. The product to be purified was made up to 50μl by adding pure water (Promega) and added to the column, this was centrifuged at 1800 rpm for 3 minutes

and the flow through transferred into a fresh Eppendorf and stored at -20°C. Using the High Pure columns, five times the PCR volume of High Pure binding buffer (3M guaninidine-thiocynanate, 10mM Tris-HCl and 5% ethanol (v/v); pH 6.6) was added and the mixture added to the spin column. This was centrifuged at 8000 rpm for 2 minutes, and the flow through added back to the column. This step was repeated to ensure maximal binding of the PCR product to the column, and the flow through discarded. The column was washed with 500μl High Pure wash buffer (20mM NaCl, 2mM Tris-HCl and 80% ethanol; pH 7.5), centrifuged at 8000 rpm for 2 minutes and the flow through discarded, this step was repeated using 200μl wash buffer. The column was centrifuged at 12,000 rpm to ensure the column was completely dry before 50μl pure water (Promega) was added and the product eluted by centrifugation.

2.7.3 Cloning of the cDNAs into Eukaryotic and Prokaryotic Vectors

Different vectors were used depending whether the contructs were to be used for expression of recombinant protein in prokaryotic cells or transient transfections into eukaryotic cells.

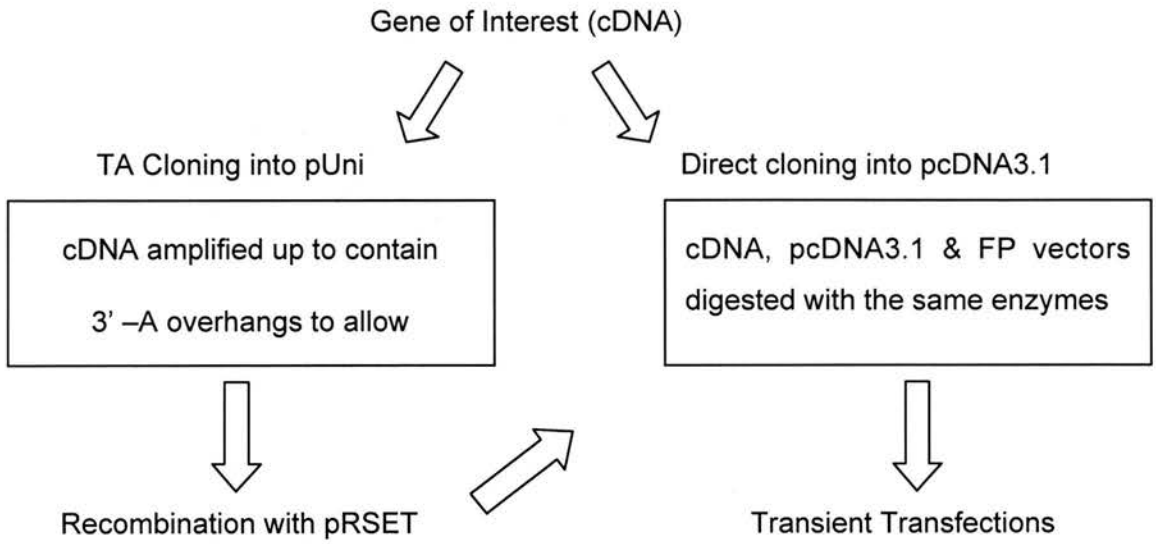
Table 2.8. Vectors used for each ER construct.

Vector	Insert	Experiment
pUni	hERβ1 & ERβ2, MmERβ1 & ERβ2, McERβ1 & ERβ2	Cloning and recombination
pRSET	hERβ1 & ERβ2, MmERβ1 & ERβ2, McERβ1 & ERβ2	Prokayotic protein expression
pcDNA3.1	hERα, ERβ1 & ERβ2, MmERβ1 & ERβ2, McERβ1 & ERβ2	Transient transfections
FP	hERα, ERβ1 & ERβ2	Protein expression

pUni, pRSET and pcDNA3.1 purchased from Invitrogen. FP vectors purchased from Clontech.

2.7.3.1 Sequence of Cloning Reactions

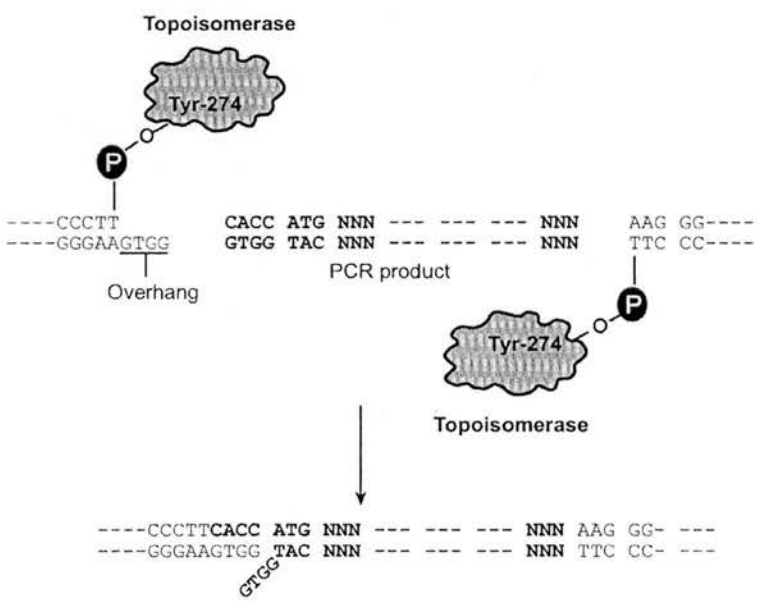
Figure 2.1. Flow diagram to show the sequence of cloning reactions performed.



2.7.3.1.1 TA Cloning of cDNA into the pUni Vector

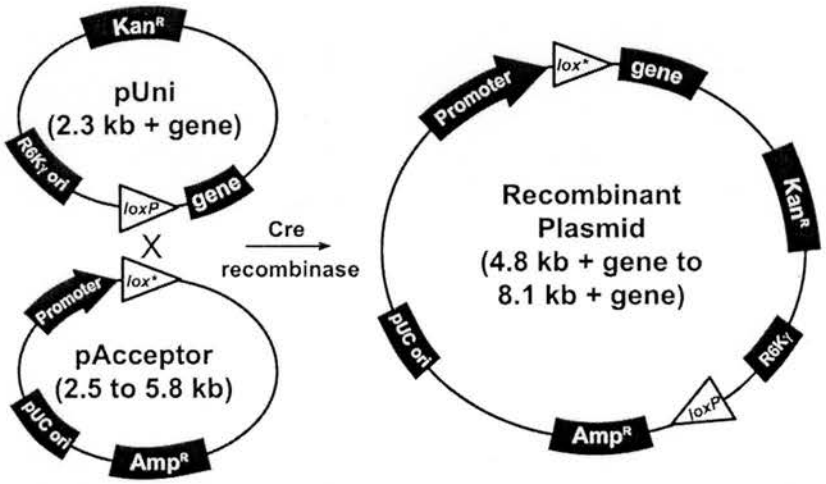
The amplified PCR product was inserted into the pUni vector into the sites shown in *Figure 2.2.*

Figure 2.2. A diagram to show the TA cloning site in the pUni vector which the PCR product was inserted into. Taken from Invitrogen product information.



2.7.3.1.2 Recombination of pUni into the Acceptor Vector pRSET

Figure 2.3. Diagram to demonstrate how pUni and the gene of interest recombines with an acceptor to produce the recombined plasmid. Taken from Invitrogen product information.



*lox** = *loxP* or *loxH* depending on acceptor vector

Once the cDNA had been cloned into the pUni vector it was recombined with an acceptor vector in the presence of Cre recombinase (*Figure 2.3*). The net result of this reaction was the formation of a recombinant plasmid.

2.7.3.2 Preparation of ERβ1/ERβ2 Expression Constructs, Cloning into pUni/V5-His TOPO (Invitrogen)

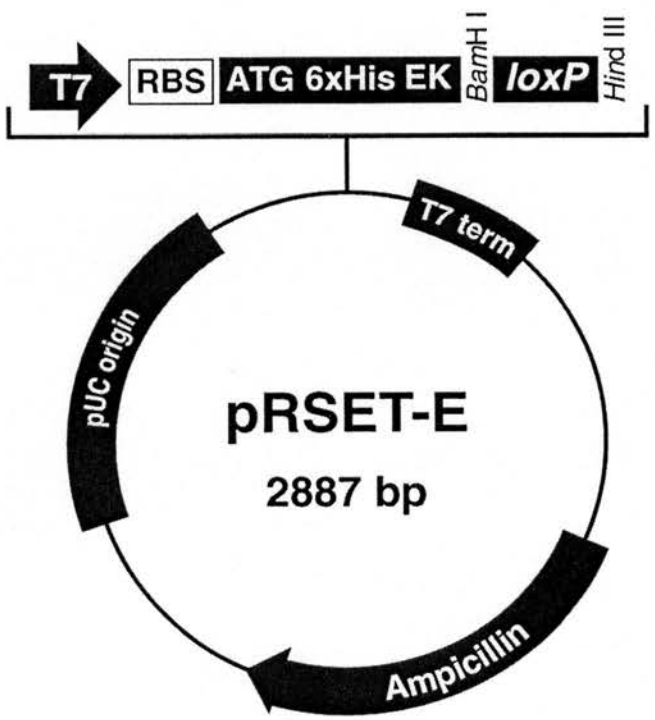
The ECHO cloning system (Invitrogen) was used to recombine the gene of interest cDNA into a pUni vector (*Figure 2.2*), therefore creating a donor vector which was used for subsequent recombinations with an ECHO adapted acceptor vector such as pRSET.

Full length ERβ1/ERβ2 cDNAs were generated from human, macaque and marmoset testes (section 2.7.1) and cloned into the pUni "donor" vector (pUni/V5-HisTOPO® Echo Cloning System) following the guidelines outlined in Invitrogen's ECHO cloning system. Specific primers (2.5.1) were used to amplify up the cDNA to enable TA cloning into pUni's specific sites. The cDNA was purified as section 2.7.2 before cloning. A 2μl volume of the purified cDNA (approximately 100ng) was mixed with 1μl NaCl (supplied by Invitrogen) and made up to 5μl with sterile water (Invitrogen). To this, 1μl TOPO® vector (Invitrogen) was added and the reaction incubated at room temperature for 15 minutes. The TOPO® reaction was

transformed into "One Shot PIRI" cells (Invitrogen) on ice. Briefly, 2μl TOPO® reaction was added to the cells and incubated on ice for 30 minutes. The cells were heat shocked at 42°C for 30 seconds to facilitate entry of the construct into the cells. The cells were returned to ice and 250μl SOC medium (2% bacto-tryptone, 0.5% bacto-yeast extract, 10nM NaCl, 2.5 mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose) was added, after which they were shaken for 1 hour at 37°C at 225 rpm. A 50μl volume was removed and spread onto Luria Bertani (LB; 10b 1^{-1} bacto-tryptone, 5g 1^{-1} bacto-yeast extract, 5g 1^{-1} NaCl pH 7.0 with NaOH) agar plates containing 50μg/ml kanamycin and left in a 37°C incubator overnight. The next day several colonies were picked and propagated overnight in kanamycin-containing LB and the plasmid extracted (section 2.9). The constructs were assessed to ensure the cloning was successful (section 2.6) and glycerol stocks produced for long term storage (section 2.9.3).

2.7.3.3 Cloning into pRSET-E (Invitrogen)

Figure 2.4. Diagram of the pRSET vector. Taken from Invitrogen product information.



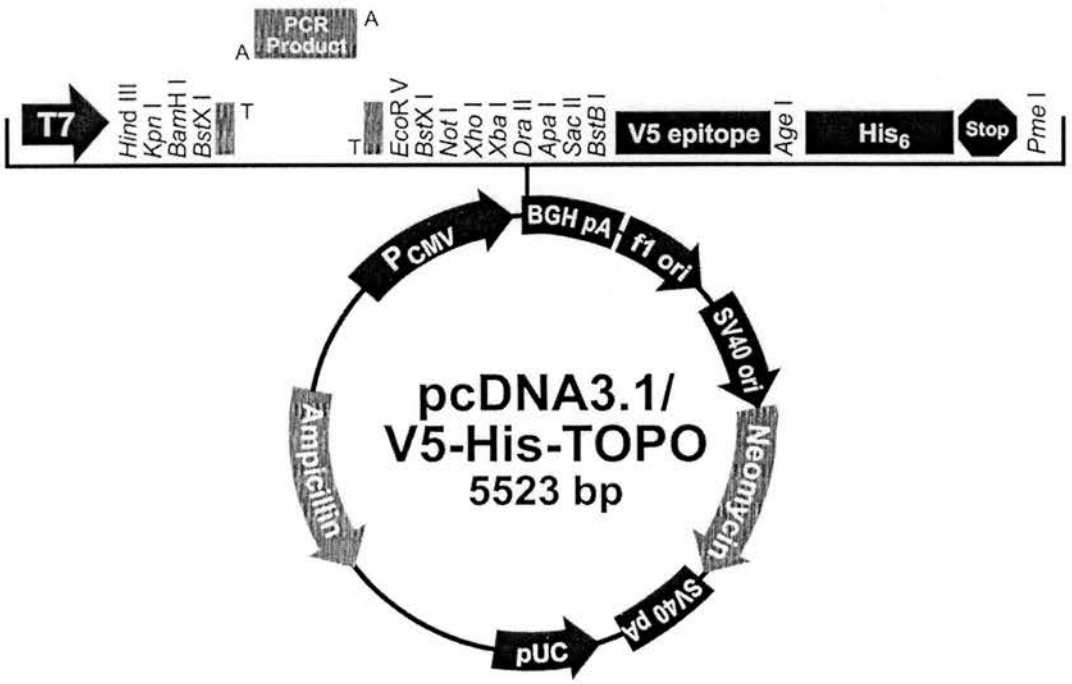
The pUni donor constructs were recombined into the pRSET-E "acceptor" vector with a T7 promoter (*Figure 2.4*) following Invitrogen's protocol. Recombination was

performed by mixing approximately 100ng of the donor vector (pUni) containing the correct cDNAs of interest, with 100ng pRSET-E and 2μl Recombinase buffer (Invitrogen). This was made up to 19μl with sterile water (Invitrogen) and 1μl Cre Recombinase (Invitrogen) added. The reaction was incubated at 37°C for 20 minutes at room temperature and inactivated by heating to 65°C for 5 minutes. A Cre recombinase from Clontech was also used for recombination reactions. In brief, 50ng pRSET, 50ng cDNA/pUni, 1μl 10x Cre buffer (Clontech), 1μl 0.05% BSA (Promega), 2μl 10mM Tris (for human cDNAs) and 0.5μl Cre recombinase (Clontech) was made to a total volume of 10μl with pure water (Promega). The reaction was incubated for 20 minutes at room temperature and then at 65°C for 5 minutes to inactivate the enzymes.

A 5μl volume of the recombination reaction was transformed into "One Shot TOP10" competent *E.coli* cells (as in section 2.7.3.2). Kanamycin selective agar plates were used and the colonies propagated in kanamycin-containing LB the following day. The resulting constructs were screened for correct insertion (section 2.6).

2.7.3.4 Cloning into pcDNA3.1/V5-His TOPO (Invitrogen)

Figure 2.5. A diagram to show the pcDNA3.1 vector. Taken from Invitrogen product information.



The pcDNA3.1/V5-His (Invitrogen) allows direct ligation of the cDNA product into a plasmid vector (*Figure 2.5*). These constructs can be directly transfected into cells and their expression analysed using the Luciferase Assay (section 2.10.2.4), or protein expression from the cells (section 2.10.2.6).

Human, macaque and marmoset cDNAs (section 2.7.1) were directly cloned into the TA cloning site of pcDNA3.1/V5-His as directed by Invitrogen's guidelines. The TOPO® cloning reaction was performed as in section 2.7.3.2 and 2μl transformed into One Shot TOP10 competent *E.coli* cells (Invitrogen). Transformation of the construct was carried out as in section 2.7.3.2, and 250μl cells plated out on ampicillin-containing agar plates and incubated overnight at 37°C. Colonies were propagated as section 2.7.3.2 and the resulting constructs analysed to ensure they contained the correct insert (section 2.6). High quality caesium chloride preparations (section 2.9.2.3) were produced for use in transient transfections (section 2.10.2).

Human ERα cDNA, which had been recovered from the pIC2OH vector (American Type Culture Collection) (section 2.7.1) using BamHI and EcoRV restriction enzymes, was purified as in section 2.7.2. It was ligated into pcDNA3.1, pre cut with the same enzymes, using Ready to Go ligase (Amersham) as discussed in section 2.8.3.

2.8 Preparation of Fluorescent Protein-tagged Constructs

To be able to visualise ERβ1 and ERβ2 proteins in transfected cells the cDNAs were tagged with fluorescent proteins (FP) using vectors purchased from Clontech. These vectors were chosen so that the ER cDNA was inserted into the vector in frame with the 3' end of the EGFP (*Figure 2.5*) and DsRed (*Figure 2.6*). The resulting protein was therefore tagged with the EGFP or DsRed protein at its N-terminus. This organisation was chosen to avoid disturbing the ligand binding or AF-2 domains at the C-terminus of the steroid receptor.

Figure 2.5. Diagram of the pEGFP-C1 (green) vector. Taken from Clontech product information.

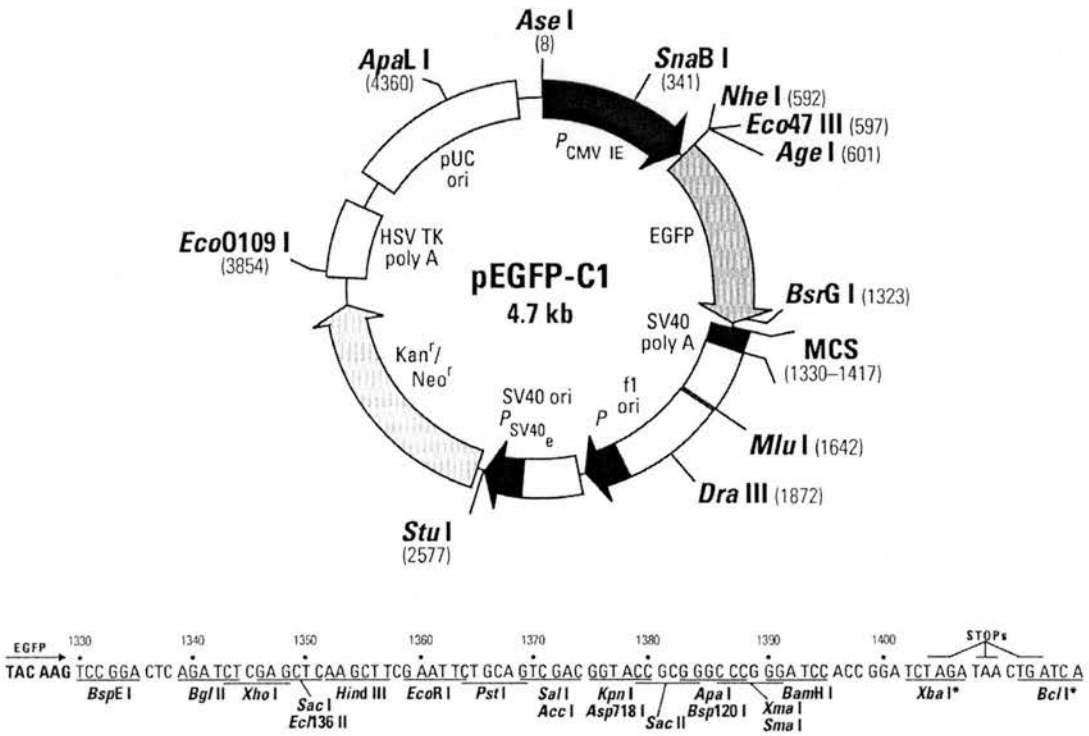
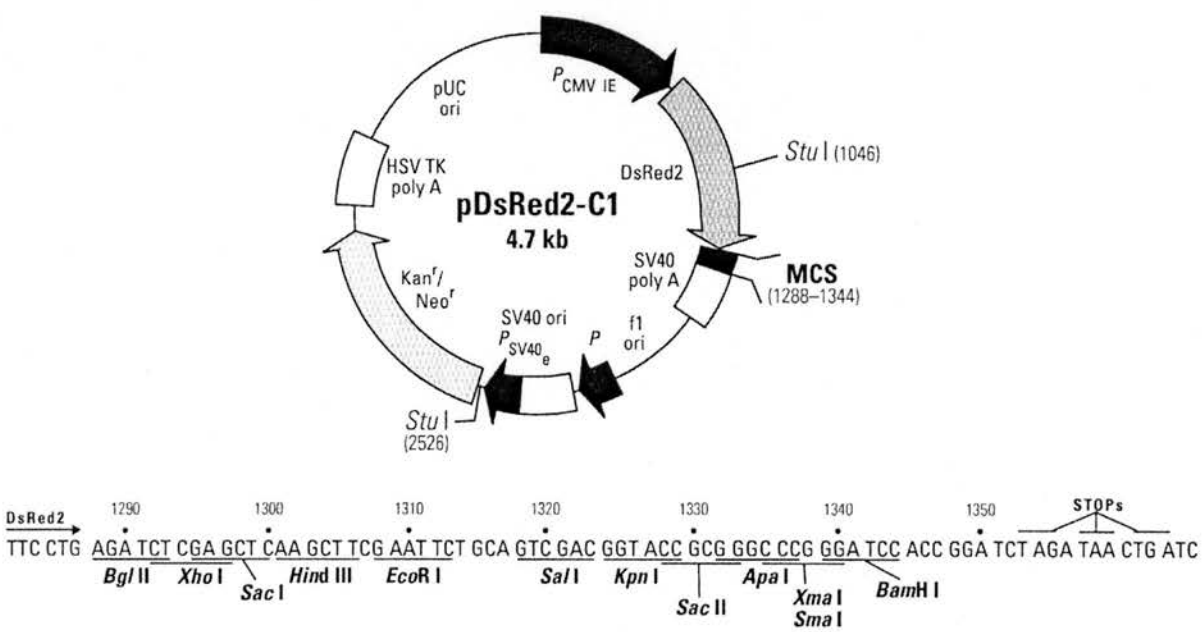


Figure 2.6. Diagram of the pDsRed2-C1 (red) vector. Taken from Clontech product information.



2.8.1 Preparation of the ER cDNAs and the EGFP and DsRed Vectors Prior to Cloning

ERβ1 and ERβ2 cDNAs from pUNI and pRSET vectors (respectively) were amplified by PCR (section 2.5) with ABgene Extensor annealing at 60°C for 30 seconds, using sequence specific primers (section 2.5.2.1) which include HindIII and BamHI restriction sites. The net result was the generation of cDNAs with HindIII and BamHI sites upstream from the start site and downstream of the stop codon of the receptor cDNA. The resulting cDNA was purified through the High Pure Purification Columns (Roche) as section 2.7.2.

2.8.2 Restriction Digestion of the cDNA and FP Vectors

The EGFP and DsRed vectors and the cDNAs were all digested with both HindIII and BamHI restriction enzymes (Promega), following the manufacture's instructions. In brief, 5μg sample was cut with 2.5μl of each enzyme and a total of 5μl enzyme buffer in a 50μl volume made up with pure water (Promega). These were left to digest at 37°C for approximately 4 hours to ensure complete digestion had occurred. The digested EGFP and DsRed vectors were incubated with Shrimp Alkaline Phosphatase (SAP) to ensure the vector would not re-ligate to itself. For every 1μg DNA present, 1μl SAP (Promega) and 1μl SAP buffer was added, this was incubated at 37°C for 15 minutes followed by de-activation by heating to 65°C for 20 minutes. Products were purified through High Pure Columns (Roche) as section 2.7.2.

2.8.3 Ligations of ERβ1/ERβ2 in the FP Vectors

Using the calculation below to ensure the correct ratio of insert to vector was used, ligations were performed to insert the cut and purified ERβ1 and ERβ2 cDNAs into the cut and purified FP vectors (section 2.8.2).

$$\frac{\text{10ng vector x insert size}}{\text{vector size}} \times \frac{3}{1}$$

The Ready-to-Go T4 DNA ligase mix (Amersham) was purchased already aliquoted into tubes. These tubes were mixed using a vortex and briefly centrifuged before use. Approximately 63ng ERβ1 or ERβ2 was added to 37ng of the FP vector. The volume was made up to 20μl with pure water (Promega) and the mix added to the

Ready-to-Go T4 DNA ligase tube. The ligation mix was then incubated at room temperature for 5 minutes and then at 16°C for 45 minutes, with occasional mixing using a vortex.

2.8.4 Transformations of the Ligated Products

Transformations were carried out by adding 2μl of the ligation mix (approximately 10ng DNA) (section 2.8.3) to 50μl competent XL1 Blue cells (Stratagene) in a chilled tube and incubated on ice for 30 minutes. The plasmid was introduced into the cells by heat shocking the tube at 42°C for 35 seconds. The cells were returned to ice for two minutes, before 450μl SOC medium was added. The cells were incubated with vigorous shaking (225 rpm) for 1 hour at 37°C, after which all of the 500μl cells/SOC medium was spread onto LB agar plates containing 50μg/ml kanamycin, as the FP vectors contain the kanamycin resistance gene. Plates were then inverted and incubated at 37°C overnight when bacterial colonies appeared. Colonies were propagated in 5ml kanamycin-containing LB overnight and a "mini plasmid prep" performed to obtain the plasmid (section 2.9.2).

2.8.5 Plasmid Analysis

To ensure ERβ1 or ERβ2 cDNAs had been inserted correctly into the FP vector, the plasmids obtained from the colonies grown overnight (section 2.8.5) were either linearised with one or digested with both HindIII and BamHI enzymes as previously (section 2.8.2). The cut products were then run on an agarose gel (Section 2.6.1). If the plasmid appeared to contain an insert of the appropriate size the plasmid was sequenced (section 2.6.3) to confirm that no mutations had been introduced during the PCR amplification or cloning reaction.

2.9 Plasmid Preparation

2.9.1 Plasmid Propagation

Plasmid stocks were prepared by picking a single colony off the agar plate and propagating it overnight by shaking at 225 rpm at 37°C in 5ml LB broth containing 50μg/ml ampicillin or kanamycin depending on which resistance gene the vector contained.

2.9.2 Plasmid Extraction

Three different methods were used to isolate the plasmid from the bacterial cultures, the first method was using a kit purchased from Promega for purification of products used for cloning and the second method was a crude preparation used to determine if reactions such as cloning had been successful. The caesium chloride (CsCl) preparation was a method used to obtain high quality DNA for transient transfections.

2.9.2.1 Wizard ® Plus SV Minipreps DNA Purification System (Promega)

Plasmid DNA was isolated from 1.5ml of the bacteria culture using the alkaline lysis method of the Wizard ® Plus SV Minipreps DNA Purification System. Briefly, the culture was centrifuged for 1-2 minutes at 14,000 rpm and the pellet resuspended in 250μl "Resuspension solution" (50mM Tris-HCl pH 7.5, 10mM EDTA and 100μg/ml RNase). The tube was inverted a couple of times, after addition of 250μl "Cell Lysis solution" (0.2M NaOH and 1% SDS) to lyse the cells and denature the chromosomal DNA. 10μl alkaline protease solution added and the contents again mixed by inverting. The tube was left to incubate for 5 minutes at room temperature, after which 350μl "Neutralisation solution" (1.32M KOAc and 6.4% glacial acetic acid, pH 4.8) was added. After inverting the tube again it was centrifuged at 14,000 rpm for 10 minutes, resulting in the sedimentation of bacterial genomic DNA, protein and cell debris. The cleared lysate containing the plasmid DNA was passed through a spin column by centrifuging at 10,000 rpm for 1 minute. The column was washed by adding 500μl "Wash solution" (200mM NaCl, 20mM Tris-HCl, pH 7.5, EDTA and 47.5% v/v ethanol) and centrifuging at 10,000 rpm, the flow through was discarded. This step was repeated and the column centrifuged at 14,000 rpm to dry. The spin column was transferred to a sterile 1.5ml tube and 50μl pure water (Promega) was added to the spin column. This was allowed to sit for 5 minutes to allow for maximum elution of the DNA from the spin column into the water. The tube was centrifuged for 1-2 minutes at 14,000 rpm, the DNA/water was spun through the column for a second time to allow for complete elution. The plasmid flow through was transferred into a fresh tube and stored at –20°C until analysis.

2.9.2.2 Crude Alkali Preparation

The Crude Alkali Preparation method was taken from (Sambrook *et al.*, 1989) and modifications of the methods by (Birboim, 1979; Burke, 1981). The bacterial pellet was re-suspended in 100µl of ice cold Solution 1 (50mM glucose, 25mM Tris-HCl, pH8.0 and 10mM EDTA, pH 8) by vigorously vortexing. To this, 200µl freshly prepared Solution 2 (0.2M NaOH and 1% SDS) was added. The contents of the tube were mixed by inverting several times. The tube was placed on ice and 150µl ice cold Solution 3 (5M KOAc, glacial acetic acid and water) added. The tube was mixed using a vortex to ensure Solution 3 was thoroughly dispersed through the viscous bacterial lysate. The tube was left to stand on ice for 5 minutes before being centrifuged at 12,000 rpm for 5 minutes at 4°C. The supernatant was transferred to a fresh tube and the pellet discarded. An equal volume of phenol:chloroform was added and the solution mixed using a vortex. The tube was centrifuged at 12,000 rpm for 2 minutes at 4°C and the top aqueous layer transferred to a fresh tube. The double stranded DNA was precipitated by adding 2 volumes of 100% ethanol to the tube and mixing with a vortex before being left to stand at room temperature for 2 minutes. The tube was centrifuged at 12,000 rpm for 5 minutes at 4°C and the supernatant removed. The pellet was washed with 500µl 70% ethanol at 4°C and the tube centrifuged as in the previous step. The pellet was left to air dry before being re-suspended in 50µl TE (pH 8.0) or 50µl pure water (Promega) and stored at –20°C.

2.9.2.3 CsCl Preparations

CsCl preparation of plasmids yields a high quality pure DNA which were used for transfection studies. The method was taken from Sambrook *et al.*, (1989).

2.9.2.3.1 Plasmid DNA Preparation

Bacterial culture was grown in a 10ml volume overnight at 37°C and transferred into a larger 500ml volume over a second night at 37°C. The culture was split into two centrifuge flasks (250ml in each) and centrifuged at 6000 rpm at 4°C for 10 minutes. The supernatant was discarded and the pellet re-suspended in 36ml solution P1 (50mM glucose, 25nM Tris-HCl pH 8.0 and 10mM EDTA). To lyse the cells, 36ml solution P2 (0.2M NaOH and 1% SDS) was added and the cell solution thoroughly mixed. The SDS in the P2 solution complexes with proteins present. Solution P3

(3M KOAc pH 4.8) was chilled prior to use and 36ml added to the cells, thus precipitating the proteins and high molecular weight DNA. This mixture was left on ice for 15 minutes or longer, before centrifuging at 7000 rpm at 4°C for 15 minutes. The clear and glassy looking supernatant was immediately filtered through Miracloth (Calbiochem) into a clear centrifuge tube. To the supernatant, 0.7 volumes of Isopropanol was added and the solutions gently mixed, the tube was then centrifuged at 7000 rpm at 4°C for 15 minutes. The supernatant was discarded and the pellet re-suspended in 4 ml TE. To this, 2ml Tris saturated phenol was added and the tube inverted vigorously several times, 2ml chloroform was added and the tube inverted vigorously again. The tube was centrifuged at 2500 rpm for 5 minutes. The aqueous layer was transferred to a fresh tube and 0.1 volume of 3M NaOAc, pH 5.0 was added, followed by 2 volumes of 100% ethanol. The solution was mixed and left at -20°C for at least 15 minutes to allow for the precipitation of the DNA. The tube was centrifuged at 8000 rpm at 4°C for 5 minutes. The supernatant was removed and the DNA pellet washed with 70% ethanol and centrifuged again. The pellet was dried and resuspended in 2ml TE.

2.9.2.3.2 Preparation of CsCl Gradient

The gradient was prepared in a 2ml Eppendorf tube. To the tube, 1.7g CsCl and 40μl ethidium bromide (10mg/ml) was added, along with 1.4ml of the DNA sample (section 2.9.2.3.1). This mixture was transferred to a 2ml polyallomer Re-Seal™ tube (Kendro Laboratory), which was sealed with a plug and crown. The tube was centrifuged in a Sorvall microultracentrifuge (Kendro Laboratory) for 2.5 hours using a gradient spin starting at 150,000 rpm decreasing to 80,000 rpm all at 20°C. The ethidium bromide intercalates with the plasmid DNA which could be visualised as a band in the middle of the tube, with the RNA forming a pellet at the bottom of the tube.

2.9.2.3.3 Recovery of Plasmid DNA

An 18 gauge needle was used to pierce the neck of the tube to allow air to pass freely into the tube. Another needle was used to pierce the tube just below the DNA plasmid band and a hypodermic syringe used to extract out the contents of the band which was then transferred into two fresh tubes.

2.9.2.3.4 Removal of Ethidium Bromide

Following transfer of the plasmid DNA an equal volume of CsCl-saturated isopropanol was added and the contents shaken vigorously. TE was added if the CsCl came out of solution. The organic and aqueous phases were left to separate and the upper aqueous phase discarded. This process was repeated until the two phases were completely clear. The lower organic phase was then transferred to a fresh tube and 0.1 volume of 3M NaOAc, pH 5.0, and three volumes of 70% ethanol added. This was gently mixed and precipitated at -20°C for 45 minutes or -70°C for 20 minutes. The tube was centrifuged at 7000 rpm at 4°C for 10 minutes and the supernatant discarded. A 500μl volume of 70% ethanol was added to the pellet and the contents of the tube mixed using a vortex before being centrifuged at 13,000 rpm for 10 minutes. The pellet was air dried and re-suspended in 1ml TE. To this, 250μl Tris-saturated phenol was added and the solution inverted vigorously, 250μl chloroform was added and the tube inverted again. The tube was centrifuged at 13,000 rpm for 5 minutes. The aqueous phase was transferred to a new tube and 500μl chloroform added and inverted vigorously again and the tube centrifuged as before. The aqueous phase was aliquoted to a fresh tube and 0.1 volume 3M NaOAc; pH 5.0 and 3 volumes of 100% ethanol added. The solution was precipitated at -20°C for 30 minutes and centrifuged at 13,000 rpm for 10 minutes. The DNA pellet was washed in 70% ethanol, centrifuged and left to air dry. The pellet was finally re-suspended in 500μl TE and the purity and concentration determined by running the sample on an agarose gel (section 2.6.1) and Genequant analysis (section 2.6.2).

2.9.3 Glycerol stocks

After plasmid preparation and analysis to ensure that the insert size, orientation and sequence was correct, a stock of *E.coli* from which fresh plasmid could be recovered at a later date was prepared. An 800μl aliquot of the bacterial culture was added to 300μl 100% glycerol mixed using a vortex and stored at -70°C.

2.10 Cell Culture and Transfections

All cell culture work was carried out in a class 2 cabinet. Cells were maintained in an incubator at 37°C with 5% carbon dioxide.

2.10.1 Cell Culture

Cells were maintained in DMEM Phenol Red Free Media (Sigma, Catalogue Number; D-5921) supplemented with 10% charcoal-stripped fetal bovine serum (Gibco, Catalogue Number; 10106), 1% D-(+)-Glucose (45% solution, Sigma. Catalogue Number; G-8769), 1% 2mM L-Glutamine (Sigma, Catalogue Number; G-7513), 1% Non-Essential Amino Acids (Ambion), 1% Fungizone (Gibco) and 1% Penicillin-streptomycin (Sigma, Catalogue Number; P-4333). For transfections, the media contained the above, except the fetal bovine serum and antibiotics were omitted.

The cells were split using 1x trypsin as in section 2.3.1. Briefly, the media was removed from the cells in the 75ml flask, 2ml trypsin was added to cover the cell layer and the flask incubated at room temperature until the cells had detached. Media was added to neutralise the trypsin, making the volume up to 10mls and the cells were counted using a haemocytometer so that they could be plated out at the correct density.

2.10.2 Transient Transfections

The cell line to be used was plated out at the densities given in *Table 2.9*, 24 hours prior to transfection. Two transfection reagents were used to introduce the DNA into the cells; either Gene Porter (Gene Therapy Systems) or JetPEI (Cambridge Biosciences).

Plates and Dishes Used:

- 12 well plates (Costar) for Luciferase assays (section 2.10.2.4)
- 35mm glass bottom microwell dishes (Plastik® Cultureware, MatTek Coporation) for confocal microscopy (section 2.10.2.5)
- 60mm dishes (Cellstar ® Greiner bio-one) for protein extraction (section 2.10.2.6)
- Chamber well slides, 8 well (Nalgene Nunc International) for cell immunocytochemical analysis (section 2.10.2.7)

Table 2.9. Details of the cell numbers plated onto different dishes.

Dish/Cell line	12 well plate	Confocal dish	60mm dish	Chamber well
Hep G2	$1x10^5$cells/well	$2x10^5$cells/dish	$4x10^5$cells/dish	$0.3x10^5$cells/well
Hek 293	$1.5x10^5$cells/well	$3x10^5$cells/dish	$6x10^5$cells/dish	$0.5x10^5$cells/well

2.10.2.1 Gene Porter

In brief, transfection media was aliquoted into two tubes according to the type of dish to be used, using the volumes given in *Table 2.10*. Plasmid DNA was added to one of the aliquots of media and Gene Porter was added at a ratio of 6μl Gene Porter to 1μg of plasmid DNA, to the other aliquot. These aliquots were mixed together and incubated at room temperature for 45 minutes to allow for complexes between the DNA and Gene Porter to occur. This was then added to the cells in the media-free wells.

Table 2.10. Details of the volumes and concentrations used in transfections.

Reagent	12 well plate	Confocal dish	60mm dish	Chamber well
Gene Porter (Total vol.)	500μl media	-	-	-
JetPEI (Total vol.)	100μl 150mM NaCl 400μl media on cells	250μl 150mM NaCl 750μl media on cells	500μl 150mM NaCl 1.5ml media on cells	50μl 150mM NaCl 200μl media on cells
DNA added (Total)	1.3μg	2μg	6μg	0.5μg

2.10.2.2 JetPEI

The plasmid DNA was mixed with 150mM NaCl (volume determined by size of dish) and a 2:1 ratio of JetPEI to μg of plasmid DNA added to another aliquot of 150mM NaCl (*Table 2.10*). These two aliquots were mixed together and left to incubate at room temperature for 30 minutes before being added to cells with transfection media present. See *Table 2.10* for volumes.

Four hours post transfection, a 1x volume of complete media was added to the wells with an additional 10% charcoal stripped fetal bovine serum and the specified ligand. Twenty-four hours after transfection the media was removed from the cells and fresh media was added along with the ligand the cells had previously been incubated with. The cells were harvested 48 hours after transfection using Promega's Lysis Buffer as manufacturer's instructions. All experiments were carried out in duplicate or triplicate and repeated a minimal of three times on separate occasions.

2.10.2.3 Constructs for Transient Transfections

2.10.2.3.1 Oestrogen Response Elements (EREs)

The EREs used were tagged to the luciferase promoter construct so their response to ER activation in transient transfections could be analysised using the Luciferase Assay (section 2.10.2.4). The Oxytocin ERE was obtained from Norbert Walther (Institute for Hormone and Fertility Research, University of Hamburg, Germany (Adan *et al.*, 1991)) already tagged to the luciferase promoter and cloned into the pGL2 Basic Vector. The 3x ERE-TK-Luc reporter construct was a gift from S.C. Nagel and D.P. McDonnell (Department of Pharmacology and Cancer Biology, Duke University Medical Centre, North Carolina, USA. (Nagel *et al.*, 2001)).

The vitellogenin ERE sequence (Klinge, 2001) was cloned into pGL2-Basic-Luciferase vector (Promega), and the pTAL-Luciferase vector (Promega) to form Vit ERE (pGL) and pTAL ERE respectively (work performed by Graeme Scobie, MRC Human Reproductive Sciences Unit).

2.10.2.3.2 Oestrogen Receptors

Human, Macaque and Marmoset full length ERβ1 and ERβ2 that had been cloned into pcDNA 3.1 (section 2.7.3) and Human ERα (section 2.7.3) were used for transient transfection.

2.10.2.3.3 SRC-1 Preparation

SRC-1e and SRC-1a constructs were a kind gift from M. Parker (Molecular Endocrinology Laboratory, Imperial Cancer Research Fund, London. (Kalkhoven *et al.*, 1998)) supplied in the pSG5 vector. The constructs were reconstituted in TE, transformed into XLI Blue cells (Stratagene) and plated onto ampicillin-containing

agar plates (section 2.8.5). Prior to use in transient transfection studies the plasmids were purified using the CsCl technique (section 2.9.2.3).

2.10.2.3.4 Renilla Internal Control

The pRL-CMV vector (Promega) contains the CMV enhancer and early promoter elements which provide a high level expression of Renilla luciferase. When co-transfected with the ERE-luciferase constructs this provides an internal control for the luciferase assay. The pRL-CMV vector was transformed into XL1 Blue cells (Stratagene), plated out onto agar plates and propagated in LB (section 2.9.1). A CsCl preparation (section 2.9.2.3) was performed so that the construct was ready for transfections.

2.10.2.4 Luciferease Assay

The Dual-Luciferase® Reporter Assay (Promega) was used to measure the level of transcriptional activity. The experimental ERE-Luc reporter activity was corrected against the Renilla internal control. This normalisation of the luciferase activity was essential as it minimises the experimental variability caused by the differences between cell viability or transfection efficiency. The results were expressed as a fold increase in activity over the control, ie. the luciferase value given for constructs transfected into the cells in the absence of ligand.

2.10.2.4.1 Harvesting the Cells

The media was removed from each of the wells in the 12 well plate and 250μl 1x Passive Lysis Buffer (Promega) added. The plate was gently rocked at room temperature for 15 minutes to ensure the cells were lysed. Using a pipette, the cells were aspirated from the wells and into an Eppendorf. Where necessary, the cells were scraped from the plate using a cell scraper (Nalgene Nunc International). The tube of cells was then left to sit at room temperature for a further 5 minutes to ensure complete lysis, before being centrifuged at 14,000 rpm at 4°C for 2 minutes.

2.10.2.4.2 Assaying the Cell Lysate Solution

The Luciferase Assay Buffer II was added to the Luciferase Assay Substrate (lyophilized product) in the glass bottle and briefly mixed using a vortex. A 200μl volume of Renilla Stop & Glo ® Reagent was added to the Stop & Glo ® Substrate

(dried product) in the small vial and mixed for 10-20 seconds using a vortex. This was then added to the Stop & Glo ® Substrate Solvent.

The microplate luminometer (Berthold Laboratories) was primed through injector M, with the Luciferase Reagent before being programmed to read the samples. Each of the cell lysate samples was aliquoted in 20μl volumes in duplicate into a white 96 well plate (Costar). The plate was read and the data generated saved for analysis.

Once all of the samples had been read for their Luciferase activity, the luminometer was washed with water and injector M was primed with the Renilla Reagent. The plate was read again, exactly as for the Luciferase reagent, and the data saved.

2.10.2.5 Transient Transfections for Confocal Analysis

The cells were plated out onto 35mm glass bottom microwell dishes as section 2.10.2 and transfected with the appropriate fluorescent construct. The media was removed from the cells 48 hours after transfection and the cells were washed with PBS. The cells were maintained in 1ml PBS with 25mM Hepes buffer (Sigma, Catalogue Number; H-0887) to give them a minimal amount of nutrients. The cells were then observed under a confocal microscope (LSM 510, Zeiss) on a warm plate set to 37°C and treated with the appropriate ligand diluted in PBS/Hepes to 10^{-8}M.

2.10.2.6 Transient Transfections for Protein Analysis

Cells were plated out as section 2.10.2 into 60mm dishes and transfected with the appropriate constructs (2.10.2.3).

2.10.2.6.1 Extraction of Proteins

Forty-eight hours post transfection, the cells were harvested and the protein extracted using three different methods. The RIPA extraction (method taken from Sambrook *et al.*, 1989) is a crude method which does not extract the nuclear proteins, the Crude Whole Cell Protein Extraction (Maudsley *et al.*, 2000) method extracts the nuclear and cytoplasmic proteins and the Nuclear Extraction (Active Motif) can extract the cytoplasmic and nuclear fraction separately. Typically, there were $4x10^6$ cells present in the 60mm dish, therefore the following methods are given for this amount of cells.

2.10.2.6.1.1 RIPA Extraction

The media was removed from the cells in the 60mm dish and the cells incubated with 500µls 1x trypsin to detach them from the dish. The trypsin was neutralised with media and the cells gently centrifuged at 7000 rpm for 5 minutes. The media was removed from the cells and 400µl 1x RIPA Buffer (5x RIPA {5M NaCl, 1M Tris-HCl; pH7.4, 0.5M EDTA. 1% deoxycholate sodium, 0.1% SDS}, 50x Protease Inhibitor and water) added. The cells were mixed using a vortex and passed through a hypodermic syringe with a fine needle to shear the genomic DNA. The tube was then incubated on ice for 15 minutes before being centrifuged to pellet the cell debris at 14,000 rpm for 15 minutes at 4°C. The supernatant was aliquoted into a fresh pre-chilled Eppendorf tubes and stored at -70°C.

2.10.2.6.1.2 Nuclear Extraction

Using the Nuclear Extract kit (Active Motif) the nuclear, whole-cell or cytoplasmic extracts were obtained from cells. The cells were collected from the dishes by addition of 2.5ml ice cold PBS in the presence of Phosphatase Inhibitors (Active Motif) after the removal of media. If required, the cells were scraped from the bottom of the dish using a cell scraper (Nalgene Nunc International). Once the cells were aspirated from the dish, the cell suspension was centrifuged at 5000 rpm for 5 minutes at 4°C. The supernatant was discarded and the cells re-suspended in 250µl 1x Hypotonic Buffer (Active Motif) to swell the cell membrane and to render it fragile. The cells were pipetted up and down several times before 15µl Detergent (Active Motif) was added. The tube was mixed using a vortex for 10 seconds to ensure the cytoplasmic proteins leaked out into the supernatant. The tube was centrifuged at 14,000 rpm for 30 seconds at 4°C and the supernatant (cytoplasmic fraction) was collected and stored at -70°C. The nuclear pellet was re-suspended in 25µl Complete Lysis Buffer (Active Motif) with Protease Inhibitor Cocktail, aided by mixing with a vortex for 10 seconds. The tube was left on ice for 30 minutes on a rocking platform so that the nuclei were lysed and the nuclear proteins were solubilised. The contents of the tube were mixed with a vortex and centrifuged at 14,000 rpm for 10 minutes at 4°C, the supernatant (nuclear fraction) was transferred to a fresh tube and stored.

2.10.2.6.1.3 Crude Whole Cell Protein Extraction

The media was removed from the cells and the dish placed on ice. The cells were washed gently by adding 3ml ice cold PBS to each plate and carefully aspirating it off. A volume of 0.3ml NP40-based lysis buffer (Stock solution: 5M NaCl, 1M Hepes, 10% glycerol. 0.5% NP40 and 1M EDTA; pH 8, plus 0.5mM Na_3VO_4, 10μg/ml leupeptin and 100mM PMSF added on the day of use) was added to each dish. The dish was then rocked for 15 minutes on ice before the cells were scraped and aspirated off into an ice cold Eppendorf tube. The tube was centrifuged at 15,000 rpm for 15 minutes at 4°C and the supernatant transferred to a fresh pre-chilled tube. The sample was stored at -70°C. On day of use, an aliquot was taken and added to an equal volume of Laemmli buffer (10% SDS, 0.01% β-mercaptoethanol, 10% glycerol and 1M Tris; pH 7.0).

2.10.2.6.2 Measuring Protein Concentration

The concentration of the cell lysate was measured using the BioRad Protein Assay (Bio-Rad Laboratories). BSA protein standards (0.125mg/ml to 1.5mg/ml) were made up, using the appropriate lysis buffer according to the sample to be measured. To each ml of Reagent A (BioRad) required, 20μl Reagent S (BioRad) was added. Using a clear 96 well plate, 5μl of the standards and 5μl of each sample were aliquoted out in duplicate. To these, 25μl Reagent A/S (BioRad) was added followed by 200μl Reagent B (BioRad). The plate was then incubated under a cover to prevent evaporation, for 15 minutes at room temperature. The colour absorbance was then measured at 650nm in a plate reader (Multiscan EX Labsystems). The results were analysed by calibrating the protein samples with the standards.

2.10.2.6.3 Protein Electrophoresis, Transfer and Detection

The extracted proteins were subjected to Western analysis and their presence detected by specific antibodies. Western blotting detects specific proteins resolved using SDS-PAGE. The technique uses the specificity of antibody/protein interactions.

2.10.2.6.3.1 Polyacrylamide Gels

Protein analysis was performed using 12% gels made up as follows. The resolving gel was made up of 6ml 30% acrylamide, 3.8mls 1.5M Tris; pH 8.8, 0.15ml 10%

SDS, 0.1ml 10% APS (ammonium persulphate) and 0.01ml TEMED made up to 15mls with water. This was poured into a cassette (Invitrogen) and left to set. The 4% stacking gel consisted of 0.65mls 30% acrylamide, 0.63ml 1.0M Tris; pH 6.8, 0.05ml 10% SDS, 0.05ml APS and 0.005ml TEMED made up to 1.5ml with water. The stacking gel was poured into the cassette once the resolving gel had set and a comb inserted so that loading wells were formed. The gels were stored at 4°C. Pre-cast 12% denaturing polyacrylamide gel (Invitrogen) were also used.

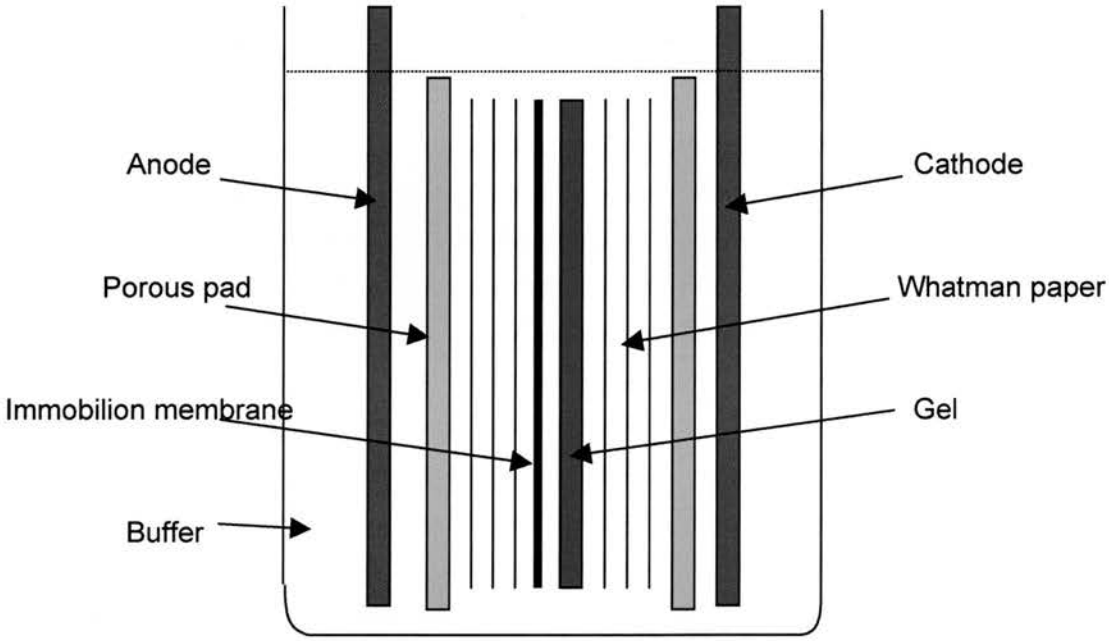
2.10.2.6.3.2 Protein Samples and Electrophoresis

A known concentration of the sample to be run on the polyacrylamide gel was mixed with 5x SDS loading dye (50mM Tris-HCl; pH 6.8, 100mM DTT (dithiothreitol) 2% SDS, 0.1% bromophenol blue and 10% glycerol with 0.01% β-mercaptoethanol) or an equal volume of Laemmli buffer (section 2.10.2.6.1.3) and boiled for 2-3 minutes. The sample was then loaded onto the gel immersed in 1x electrolyte running buffer (25mM Tris, 250mM glycine; pH 8.3 and 0.15% w/v SDS) in an X Cell Sure Lock electrophoresis tank from Novel Experimental Technology. An aliquot of pre-stained SDS-Page standards (BioRad) was run alongside the sample. The gel was run at 120 volts over approximately two hours until the dye front had run to the bottom of the gel.

2.10.2.6.3.3 Protein Transfer

Once electrophoresis was complete, proteins were transferred onto an Immobilion transfer membrane (Millipore) in blotting buffer (48mM Tris, 39mM glycine and 20% methanol) using an electrophoresis unit from Hoefer Scientific Instruments as pictured in *Figure 2.7*. This was performed either overnight run at 20 volts or over four hours run at 40 volts.

Figure 2.7. Diagram of the Western transfer apparatus.



2.10.2.6.3.4 Western Analysis

The gel was stained with Coomassie blue (0.1% w/v Coomassie blue R250 in 50% v/v methanol and 7.5% v/v acetic acid) overnight to observe whether the proteins had run properly. The transferred membrane was removed from the tank and incubated with 1% (w/v) dried semi-skimmed milk in TBST (50mM Tris-HCl, 150mM NaCl; pH 7.4, and 0.05%Tween-20) for 1 hour at room temperature. The membrane was washed with TBST and then incubated overnight at 4°C with the primary antibody made up in TBST to the correct dilution (*Table 2.11*).

Table 2.11. Details of the antibodies used in Western analysis.

	Antibody code/supplier	Raised in	Dilution
EGFP	Clontech (Catalogue Number; 8367-2)	Rabbit monoclonal	1:500
MAPK p44/42	Cell Signalling Technology, NEB. (Catalogue Number; 9101)	Mouse monoclonal	1:1000
Phosphorylated MAPK p44/42	As above (Catalogue Number; 9102)	Rabbit polyclonal	1:1000

The following day, the Coomassie dye was removed from the gel with de-stain (50% methanol and 5% acetic acid) so that the protein samples were visible. The primary antibody was washed off the membrane by washing 3 times for 20 minutes with TBST and then incubated for 2 hours with the appropriate HRP-conjugated secondary antibody in TBST at room temperature. The membrane was washed 3 times for 20 minutes with TBST, with a final wash with TBS (50mM Tris-HCl, 150mM NaCl; pH 7.4) before being subjected to ECL Plus Western Blotting Detection System (Amersham Life Sciences). The chemiluminescence was detected using X ray films (XLS, Kodak) which were developed using developer and fixer solutions.

2.11 Cell Immunocytochemistry

Cells were plated out into 8-well chamber well slides as section 2.10.2, and transfected with the appropriate construct. After 48 hours post transfections, the media was removed and the cells fixed in ice cold methanol for 10 minutes. Immunocytochemical analysis was carried out on the cells (section 2.12).

2.12 Tissue immunohistochemistry

Tissues embedded in paraffin wax blocks (Histology department, MRC Human Reproductive Sciences Unit) were cut into 5μm sections using a Lecia hand operated microtome RM 2135 (Leica). Sections were floated in ice cold water and then warm (42°C) distilled water and placed onto charged slides. To remove the wax after the slides were dry, they were immersed in xylene for 5 minutes. They were re-hydrated in decreasing percentages of alcohol (100%, 95% and 70%) and rinsed in water.

2.12.1 Retrieval

To visualise the receptor, antigen retrieval was performed as detailed in (Norton *et al.*, 1994; Saunders *et al.*, 2000). Retrieval was performed to reduce cross-linking between proteins that are formed by fixing the tissue. This was carried out by pressure cooking (Tefal) the slides in glycine/EDTA (50mM glycine and 0.01% EDTA) or Citrate.

Table 2.12. Details to show the antigen retrieval methods used.

	Buffer	pH	Time
ERβ1 (*Ab:M9*)	50mM Glycine/EDTA	3.5	5 minute pressure cook Sit for 20 minutes after
ERβ2 (*Ab:57*)	50mM Glycine/EDTA	3.5	5 minute pressure cook Sit for 20 minutes after
EGF receptor (*Ab:Sigma*)	Citrate		1 minute boiling

After retrieval, the slides were incubated in 3% hydrogen peroxide in methanol for 30 minutes to decrease endogenous peroxidase activity and therefore decrease background staining. The slides were washed in tap water and washed three times for 5 minutes in TBS (50mM Tris-HCl, 150mM NaCl; pH 7.4).

2.12.2 Avidin-Biotin Blocking

An avidin-biotin (Vector SP-2001) block was carried out to block endogenous biotin therefore reducing background. Four drops of avidin per ml of rabbit blocking serum (Diagnostics Scotland) in TBS/BSA was used on the slides and incubated for 30 minutes at room temperature, followed by three washes in TBS. The biotin (4 drops per ml TBS) was added to the slides for 15 minutes, after which the slides were washed again.

2.12.3 Primary and Secondary Antibodies

Table 2.13. Details of the antibodies used for immunohistochemistry.

	Antibody	Raised in	Dilution
ERβ1	M9, raised to P7 Saunders *et al.*, 2002a	Mouse monoclonal	1:500
ERβ2	M57, raised to P8 Saunders *et al.*, 2002a	Mouse monoclonal	1:40
EGF receptor	Sigma (Catalogue Number: E3138)	Mouse monoclonal	1:50

The slides were incubated with the appropriate dilution of the primary antibody overnight at 4°C (*Table 2.13*). The excess primary antibody was then washed off with TBS as before. The secondary biotinylated antibody was made up to the correct dilution in blocking serum accordingly with the primary antibody used and added to the slides for an hour at room temperature. This stage was excluded for the EGF receptor antibody which was detected using DAKO mouse Envision kit (section 2.13.5).

2.12.4 Horse-Radish Peroxidase

After TBS washes to remove the secondary antibody, the ABC-HRP detection system (DAKO) was used to amplify the signal obtained from the primary antibody. This ABC-HRP was added to the slides for 30 minutes at room temperature. Or Envision (DAKO) was added to the slides in place of the secondary antibody and the ABC-HRP for the EGF receptor (section 2.12.5).

The slides were washed and developed using Diaminobenzidene (DAB) substrate (1 drop/ml, DAKO, Catalogue Number; K3468). After DAB staining, the slides were counterstained with haematoxylin, differentiated in acid-alcohol and the blue colour in the nuclei or cell membrane (depending upon protein stained for) developed in Scotts tap water. The slides were dehydrated in increasing concentrations of alcohol and cleared in xylene before mounted under glass coverslips using pertex.

2.12.5 Envision

Instead of ABC-HRP and the secondary antibody, one drop of mouse Envision (DAKO, Catalogue Number; K4006) was added to the section and incubated for 30 minutes at room temperature before washing, DAB development, counterstaining and mounting as described in section 2.13.4 above.

2.13 Photomicroscopy

Tissue sections were examined using an Olympus Provis microscope (Olympus Optical Co.) and images were captured using a Kodak DCs330 camera (Eastman Kodak Co.). Captured images were stored on an Apple MacIntosh and images were compiled using Photoshop 7 (Adobe Systems).

Chapter 3

Cell Specific Patterns of Expression of Oestrogen Receptor Beta (ERβ1 and ERβ2) in Human and Primate Tissues, and in Cell Lines

3.1 Introduction

Oestrogens are essential regulators of both female and male fertility as well as playing a role in other tissues such as the cardiovascular system and bone. Oestrogen action is mediated via specific receptors (ER). The first oestrogen receptor was cloned from a human breast cancer cell line in 1986 (Green *et al.*, 1986). In 1996 a second ER was cloned, this time from rat prostate (Kuiper *et al.*, 1996) and its human homologue was cloned shortly thereafter (Mosselman *et al.*, 1996). The two receptors are known as oestrogen receptor alpha (ERα; NR3A1) and oestrogen receptor beta (ERβ; NR3A2). ERα and ERβ receptors are the products of two genes located on different chromosomes (Enmark *et al.*, 1997). Splice variant isoforms of the ERβ gene have been identified in a number of species including human (Inoue *et al.*, 1996; Lu *et al.*, 1998; Moore *et al.*, 1998; Ogawa *et al.*, 1998c), bovine (Walther *et al.*, 1999) and rodents (Chu and Fuller, 1997; Lu *et al.*, 2000; Lu *et al.*, 1998).

3.1.1 Oestrogen Biosynthesis

Oestrogen biosynthesis is dependent upon expression of the aromatase complex in females and in males. This enzyme complex is a product of the *cyp19* gene and is composed of a specific cytochrome p450 aromatase and a ubiquitous NADH-cytochrome P450 reductase (Simpson *et al.*, 1994). The aromatase complex is located in the endoplasmic reticulum of the cells and is present in numerous tissues (reviewed in de Ronde *et al.*, 2003). Cytochrome P450 is found at the highest levels in the human placenta and the granulosa cells of the mature ovarian follicle (Simpson and Davis, 2001; Turner *et al.*, 2002). In the testis aromatase has been localised to the Leydig cells, immature Sertoli cells, in the mature germ cells and spermatozoa (Payne and Youngblood, 1995; Turner *et al.*, 2002).

3.1.2 Rodent Knockout Models

A number of studies on the role played by oestrogens in reproductive function have been undertaken either using rodent models in which the oestrogen receptor is disrupted and therefore oestrogen cannot activate its intended target, or by applying selective antagonists. The advent of gene deletion has allowed the generation of mice lacking ERα (αERKO) (Lubhan *et al.*, 1993), ERβ (βERKO) (Krege *et al.*, 1998) or both ERα and ERβ (αβERKO) (Couse *et al.*, 1999; Dupont *et al.*, 2000), as well as mice lacking the ability to synthesise oestrogens due to deletion of the aromatase gene; *cyp19* (ARKO) (Fisher *et al.*, 1998). All knockout models are discussed in detail in the literature review, along with the ERE-Luciferase engineered reporter mice which demonstrate the tissues in which the ER acts upon the ERE-reporter gene (Ciana *et al.*, 2001; Nagel *et al.*, 2001).

These knockout mouse models give indications of the overall physiological effect of oestrogens acting via ERα or ERβ in rodents, but they do not shed light on cell specific patterns of receptors. The discovery of a number of ERα and ERβ splice variants in both rodent (Chu and Fuller, 1997; Lu *et al.*, 2000; Lu *et al.*, 1998) and human (Inoue *et al.*, 2000; Lu *et al.*, 1998; Moore *et al.*, 1998; Ogawa *et al.*, 1998c; Petersen *et al.*, 1998) has introduced a new level of complexity in potential cellular responsiveness to oestrogens. Sequence comparison reveals that the ERβ variants in human are not identical to rodents (Enmark and Gustafsson, 1999) therefore the rodent models may not be directly comparable to humans or primates.

3.1.3 Oestrogen Receptor Beta (ERβ) Isoforms and Localisation

The current study has focused on human ERβ1, the full length wild type receptor and the ERβ2 (also known as ERβcx) splice variant isoform, the mRNA's of which have previously been reported to have differential expression in different tissues and cell lines (Moore *et al.*, 1998; Ogawa *et al.*, 1998c). In normal tissues RT-PCR analysis suggests the highest levels of expression of mRNAs for human ERβ1 is found in testis and ovary, with lower levels of expression in the heart, brain, placenta, liver, skeletal muscle, spleen, thymus, prostate, colon, bone marrow, mammary gland, uterus and fat. In contrast, human ERβ2 mRNA was detected at highest levels in spleen, thymus, testis and ovary, with lower levels of expression in skeletal muscle, prostate, colon, small intestine, leukocytes, bone marrow,

mammary gland, uterus and fat (Moore *et al.*, 1998). Differential expression of other ERβ splice variant mRNAs; hERβ3, hERβ4, hERβΔ5 and hERβ5 has also been documented (Inoue *et al.*, 2000; Moore *et al.*, 1998; Scobie *et al.*, 2002).

In addition to normal tissues, transformed cell lines derived from prostate, uterus, bone, liver, colon, breast and ovarian tumours have been analysed for expression of hERβ isoforms (Moore *et al.*, 1998). Human ERβ1 was detected in prostate, uterine, bone, breast and ovarian tumour cell lines but not in colon and liver tumour cell lines. Human ERβ2 was detected in a wide range of cell lines including those derived from prostate, uterus, breast, and endothelial cells.

In this chapter studies are described in which the human and non-human primates are used to investigate localisation of ERβ1 wild type and the ERβ2 variant at an mRNA and protein level. The Common marmoset is a New World primate (*Callthris jacchus*) and the stump-tailed macaque is an Old World primate (*Macaca arctodies*). The experiments performed complement studies from our laboratory which have shown that these primates are good models for studying the expression of the oestrogen receptors in reproductive systems (Saunders *et al.*, 2000; Saunders *et al.*, 2001).

3.1.4 Aims

The aims of the studies in this chapter were to determine if the ERβ variant mRNAs and proteins exist in primates and if they do, whether they were similar to human or rodent variants. The aim was also to determine the specific localisation of the ERβ1 and ERβ2 variant within reproductive tissues of the human and primates.

3.2 Methods

3.2.1 Identification of mRNAs by RT-PCR

Cells and tissues were collected, total RNA extracted, oligo DT primed and cDNA synthesis performed with oligo DT primers as described in sections 2.3 and 2.4 respectively. A human cDNA testis library was purchased from BD Biosciences and used as a positive control.

3.2.1.1 Screening Primers

Specific primers were chosen, as outlined in section 2.5.1, to amplify regions of the human ERβ1 wild type and human ERβ2 variant cDNAs in order to be able to determine whether mRNAs with specific sequence homology were expressed in tissue extracts or cell lines (*Table 3.1*).

Table 3.1. PCR primers used for detection of ERβ1 and ERβ2 and the GAPDH positive control.

	5' primer	3' primer	Product size
ERβ1	GGCATCTCCTCCCAGCAGCA	CACTGAGACTGTGGGTTCTGGG	240 bp
ERβ2	GGCATCTCCTCCCAGCAGCA	CACTGCTCCATCGTTGCTTC	135 bp
GAPDH	CTGCACCACCAACTGCTTAGC	ATGCCAGTGAGCTTCCCGTTC	280 bp

3.2.1.2 RT-PCR

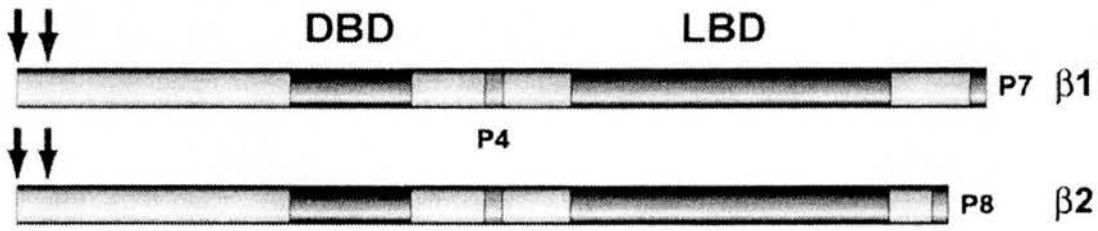
PCR was carried out using Mega Mix (Microzone) as described in section 2.5.2.3. The PCR reactions were annealed at 58°C for 30 seconds using the ERβ1, ERβ2 and GAPDH oligonucleotide primers (*Table 3.1*); DNA fragments were analysed by migration on agarose gel (section 2.6.1).

3.2.2 Antibodies for Immunohistochemical Analysis

Monoclonal antibodies to detect full length ERβ1 and ERβ2 proteins were prepared as described previously (Saunders *et al.*, 2000; Saunders *et al.*, 2002a). Briefly, the ERβ1 specific antibody (M9) was raised against the peptide P7 (CSPAEDSKSKEGSQNPQSQ) located at the C-terminus of human ERβ1 (Saunders *et al.*, 2000). The human ERβ2 antibody (M57) was raised against

peptide P8 (CMKMETLLPEATMEQ) located at the C-terminus human ERβ2 (Saunders *et al.*, 2002a) (*Figure 3.1*).

Figure 3.1 Figure to show the regions of the proteins that the antibodies were raised to. Note the P4 peptide which has been used previously to immunolocalise ERβ to human and primate tissue (Saunders *et al.*, 2000; Saunders *et al.*, 2001).



3.2.2.1 Tissue Immunohistochemistry

Marmoset tissues were previously collected from adult captive bred animals (*Callthrix jacchus*) maintained in a colony which has been closed since 1973. Tissues were also previously collected from adult stump-tailed macaques (*Macaca arctodies*, n=3). These tissues had already been fixed and embedded in paraffin wax blocks and along with tissues from human, were held in an archive in the histology section at the MRC Human Reproductive Sciences Unit (Edinburgh). Fixed sections of human endometrium were a gift from Professor Hilary Critchley and Teresa Henderson (University of Edinburgh).

Immunohistochemical studies were carried out on tissues using anti-hERβ1 and anti-hERβ2 specific monoclonal antibodies using methods described in section 2.12.

Slides were subjected to a heat induced retrieval stage (section 2.12.1) to reduce cross-linking between proteins formed during the fixation (Norton *et al.*, 1994). Sections stained for ERβ1 using the M9 antibody were pressure cooked for 5 minutes in a 50mM glycine/EDTA buffer at pH 3.5. Sections stained for ERβ2 with the M57 antibody were also pressure cooked in a 50mM glycine/EDTA buffer at pH 3.5, whereas endometrial sections were pressure cooked at pH 8.0 for 5 minutes.

An avidin-biotin block was carried out as section 2.12.2, however 6 drops per ml of blocking serum (5% NRS in TBS/BSA) were used when looking at marmoset ovaries as this tissue has high endogenous biotin activity.

The primary antibodies were diluted in NRS/TBS/BSA (anti-ERβ1 1:500; anti-ERβ2 1:40) and added to the slides after the biotin had been washed off, they were incubated overnight at 4°C (section 2.12.3).

The following day an anti-mouse biotinylated secondary antibody diluted (1:5000) as described in section 2.12.3 was incubated on slides for 1 hour at room temperature. ABC-HRP (DAKO) was used to amplify the antibody signal. This was incubated on the slides for 30 minutes before being washed off and DAB used to develop the signal. The slides were counter stained with haemotoxylin, dehydrated and mounted with pertex, then photographed as described in section 2.13.

3.2.2.2 Cell Monolayer Immunocytochemistry

Immunocytochemical analysis was performed on the cell lines (Hek 293 and Hep G2) that were used in the transient transfection studies described in Chapter 5. The cells were plated out as in section 2.10 onto chamber well slides (Nalgene Nunc International). The cells were either left to grow on their own or transfected with the ERβ-containing plasmid constructs (prepared as described in Section 2.7) as a positive control.

After 48 hours the cells were fixed in ice-cold methanol (section 2.11) and immunocytochemistry performed as section 2.12, but without the de-waxing and antigen retrieval stages. The anti-ERβ1 and anti-ERβ2 antibodies were used as described above (and in section 3.2.1).

3.3 Results

3.3.1 Detection of ERβ1 and ERβ2 mRNA in Samples from Human, Macaque and Marmoset Tissues and Cell Lines

Primers specific for ERβ1 and ERβ2 isoforms were used to amplify cDNAs from pools of cDNA prepared by RT of total RNA from cell lines and tissues and from commercially available human testis RNA (BD Biosciences). Following PCR amplification products of the expected sizes were observed on gels: ERβ1 gave a band at 249bp, ERβ2 at 135bp and GAPDH at 280bp. ERβ1 was detected in all human and marmoset tissues, only the kidney was negative (*Figure 3.1*; top panel). ERβ2 mRNA expression was detected in heart, ovary and testis in both human and marmoset (*Figure 3.1*; middle panel). However, ERβ2 mRNA was not detected in human and marmoset brain, liver, kidney or placenta. Results are summarised in *Table 3.3*.

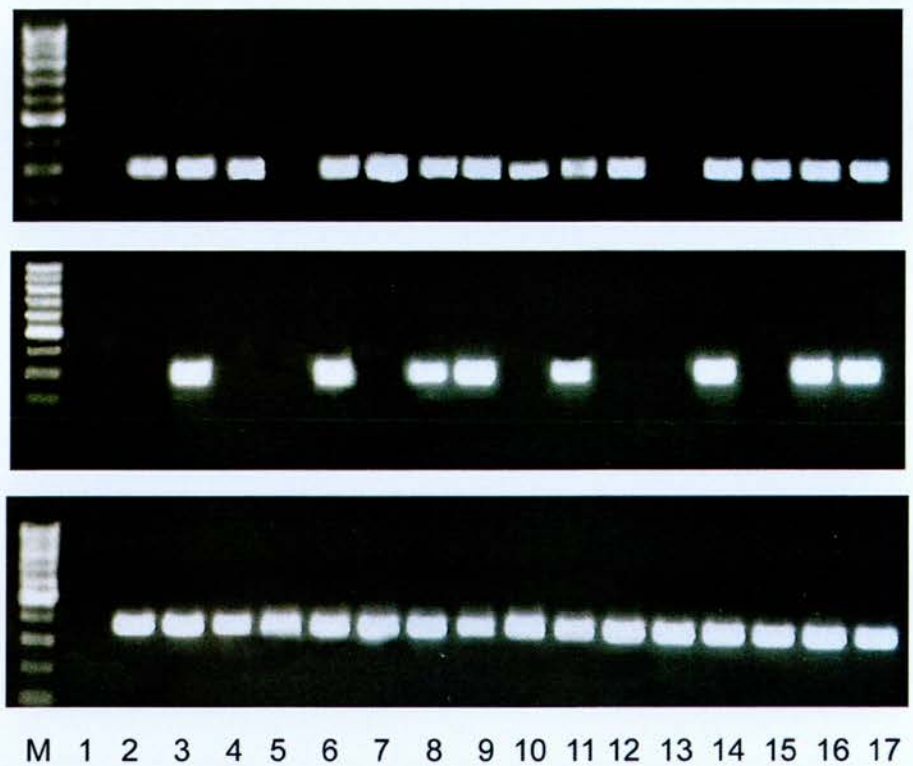


Figure 3.1. Detection of ERβ1 and ERβ2 mRNAs in cDNAs pools prepared from human, macaque and marmoset tissues. RT-PCR was performed on reproductive tissue cDNA from human, and marmoset. Specific primers were used to detect ERβ1 (top panel, 240bp), ERβ2 (middle panel, 135bp) and GAPDH (lower panel, 280bp) as a positive control. Tissues tested were (1) blank, (2) human brain, (3) human heart, (4) human liver, (5) human kidney, (6) human ovary, (7) human placenta, (8) human testis library (BD Biosciences), (9) human testis, (10) marmoset brain, (11) marmoset heart, (12) marmoset liver, (13) marmoset kidney, (14) marmoset ovary, (15) marmoset placenta, (16) marmoset testis and (17) marmoset testis. 100 bp marker was run on all gels.

Table 3.3. Summary of ERβ1 and ERβ2 mRNA expression in tissues.

Tissue	ERβ1	ERβ2
Human Brain	+	-
Human Heart	+	+
Human Liver	+	-
Human Kidney	-	-
Human Ovary	+	+
Human Placenta	+	-
Human Testis Library	+	+
Human Testis	+	+
Marmoset Brain	+	-
Marmoset Heart	+	+
Marmoset Liver	+	-
Marmoset Kidney	-	-
Marmoset Ovary	+	+
Marmoset Placenta	+	-
Marmoset Testis Pure	+	+
Marmoset Testis	+	+

RT-PCR analysis of RNA from different transformed cell lines also showed a difference in ERβ1 and ERβ2 mRNA expression (*Figure 3.2*). Hek 293, Hep G2 and Cos 7 cells were negative for both ERβ1 and ERβ2, whereas Ishikawa, LnCap and Hela cells contained detectable ERβ1 and ERβ2 mRNAs. Results are summarised in *Table 3.4*.

Figure 3.2. Detection of ERβ1 and ERβ2 mRNAs in cDNAs pools prepared from cell lines. RT-PCR was performed using specific primers to detect ERβ1 (top panel, 240bp), ERβ2 (middle panel, 135bp) and GAPDH (lower panel, 280bp) as a positive control. The cell lines tested were (1) blank, (2) Hek 293, (3) Hep G2, (4) Cos 7, (5) Ishikawa, (6) LnCap, and (7) Hela. 100 bp marker was run on all gels.

Table 3.4. Summary of ERβ1 and ERβ2 mRNA expression in cell lines.

Transformed Cell Line	ERβ1	ERβ2
Hek 293 (human kidney)	-	-
Hep G2 (human liver)	-	-
Cos 7 (monkey kidney)	-	-
Ishikawa (human endometrium)	+	+
LnCap (human prostate)	+	+
Hela (human cervical cancer)	+	+

3.3.2 Immunohistochemical Analysis of Reproductive Tissues

3.3.2.1 Immunohistochemical Analysis of the Ovary

Using the anti-hERβ1 antibody, ERβ1 protein was immunolocalised to nuclei of granulosa cells at all stages of follicular maturation, and in some cells of the corpus luteum in the human, macaque and marmoset ovaries (*Figure 3.3a, c, e*). ERβ2 immunopositive staining was detected only in the human ovary (*Figure 3.3.b*), with no staining in either the macaque or the marmoset ovaries. In the human tissue ERβ2 expression was very similar to that of ERβ1 expression, with immunopositive staining clearly visible in the nuclei of granulosa cells (G).

3.3.2.2 Immunohistochemical Analysis of the Endometrium

ERβ1 and ERβ2 proteins were detected in human endometrial tissue (*Figure 3.4*). Both ERβ isoforms were expressed in cells within the basal layer (not shown) of the human endometrium, as well as in the stromal and glandular cells of the functional layer (*Figure 3.4a, b*). In the macaque and marmoset endometrium ERβ1 immunopositive cells were also detected (*Figure 3.4c, e*). In contrast, using the anti-hERβ2 antibody, immunopositive cells were not detected in the macaque or marmoset endometrial tissues (*Figure 3.4d, f*), in contrast immunopositive cells were detected in glands and stroma of human endometrium (*Figure 3.4b*).

3.3.2.3 Immunohistochemical Analysis of the Placenta

In the human, macaque and marmoset placental tissues ERβ1 protein was detected in the nuclei of the syncytiotrophoblast and in endothelial cells surrounding the blood vessels (*Figure 3.5a, c, e*). No ERβ2 protein was immunolocalised to placental tissues from the human, macaque or marmoset (*Figure 3.5b, d, f*).

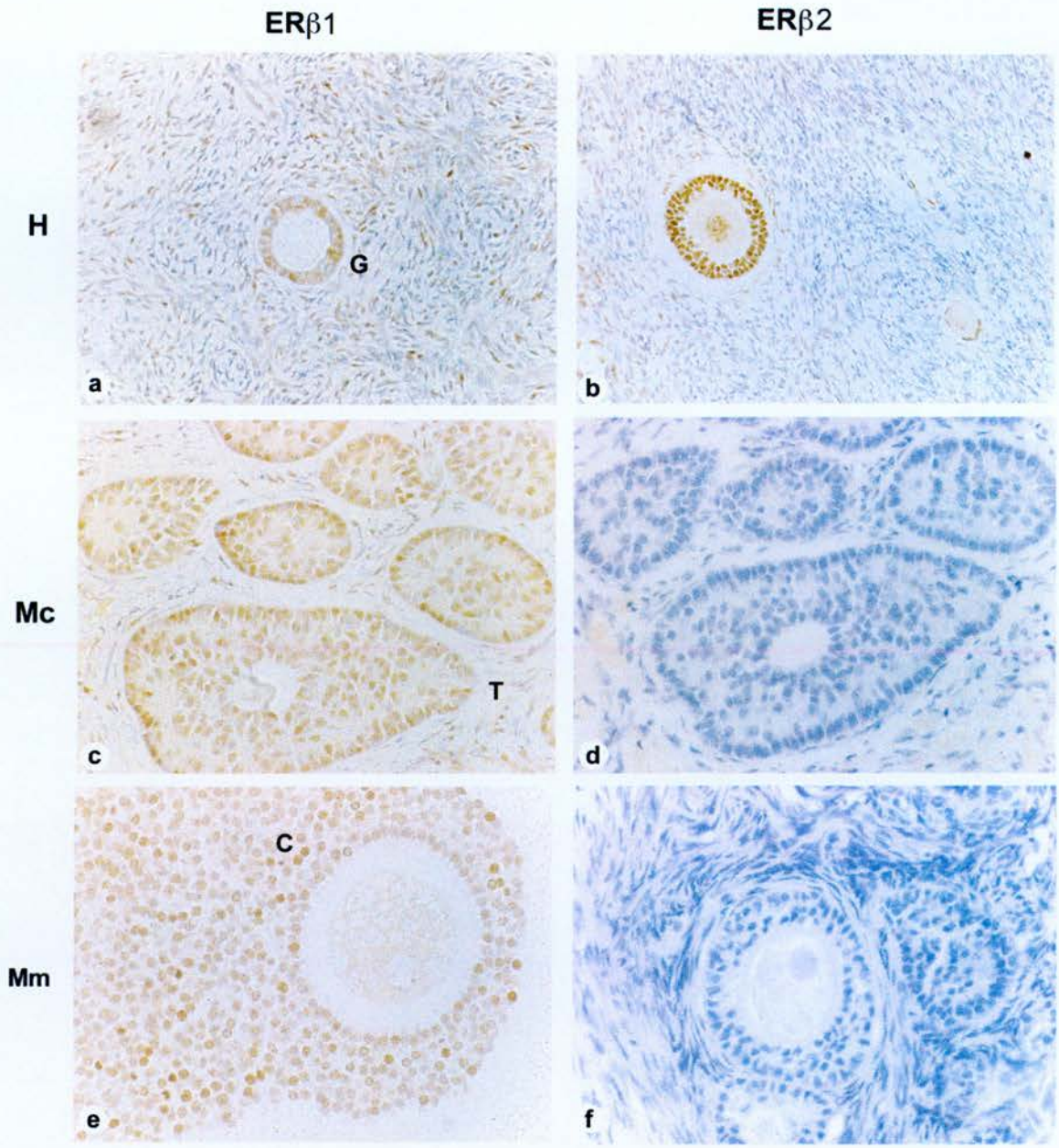


Figure 3.3. Immunohistochemical analysis of ERβ1 and ERβ2 protein expression in the ovary of human, macaque and marmoset. Sections from human (a, b), macaque (c, d) and marmoset (e, f) were stained with anti-hERβ1 or anti-hERβ2 specific monoclonal antibodies. Immunopositive staining of ERβ1 was observed in the nuclei of granulosa (G), cumulus (C) and thecal (T) cells (a, c, e). However when the same ovaries were stained for ERβ2 only the human ovarian granulosa cells were clearly immunopositive (b). Magnification is x40.

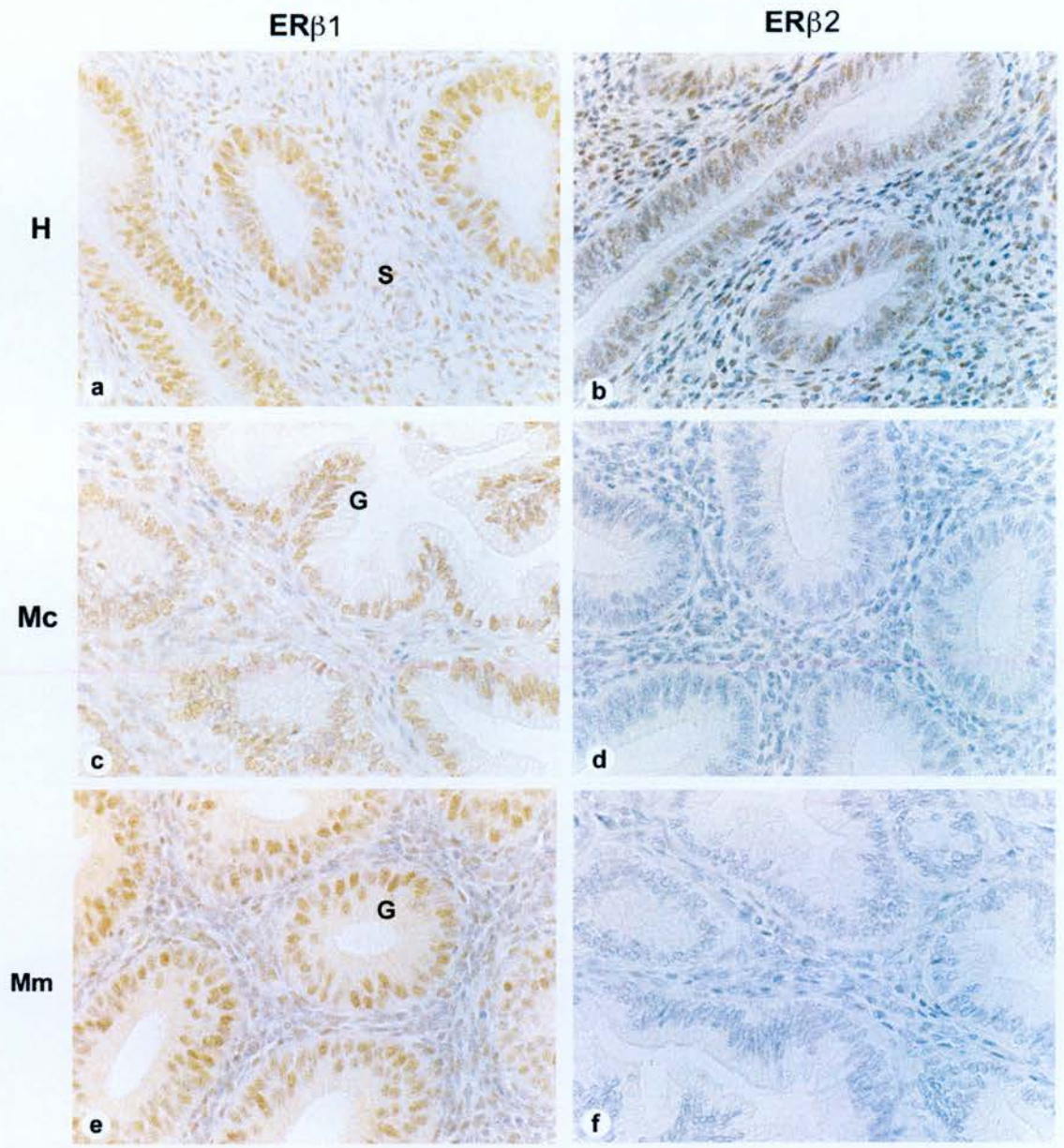


Figure 3.4. Immunohistochemical analysis of ERβ1 and ERβ2 protein expression in the endometrium of human, macaque and marmoset. Sections from human (a, b), macaque (c, d) and marmoset (e, f) were stained with the anti-hERβ1 or anti-hERβ2 specific monoclonal antibodies. Positive staining for ERβ1 was observed in the nuclei of endometrial cells both in the stroma (S) and in the epithelium lining the glands (G) (a, c, e). When the endometrial tissues were stained for ERβ2 protein only human endometrium was immunopositive (b). Magnification is x40.

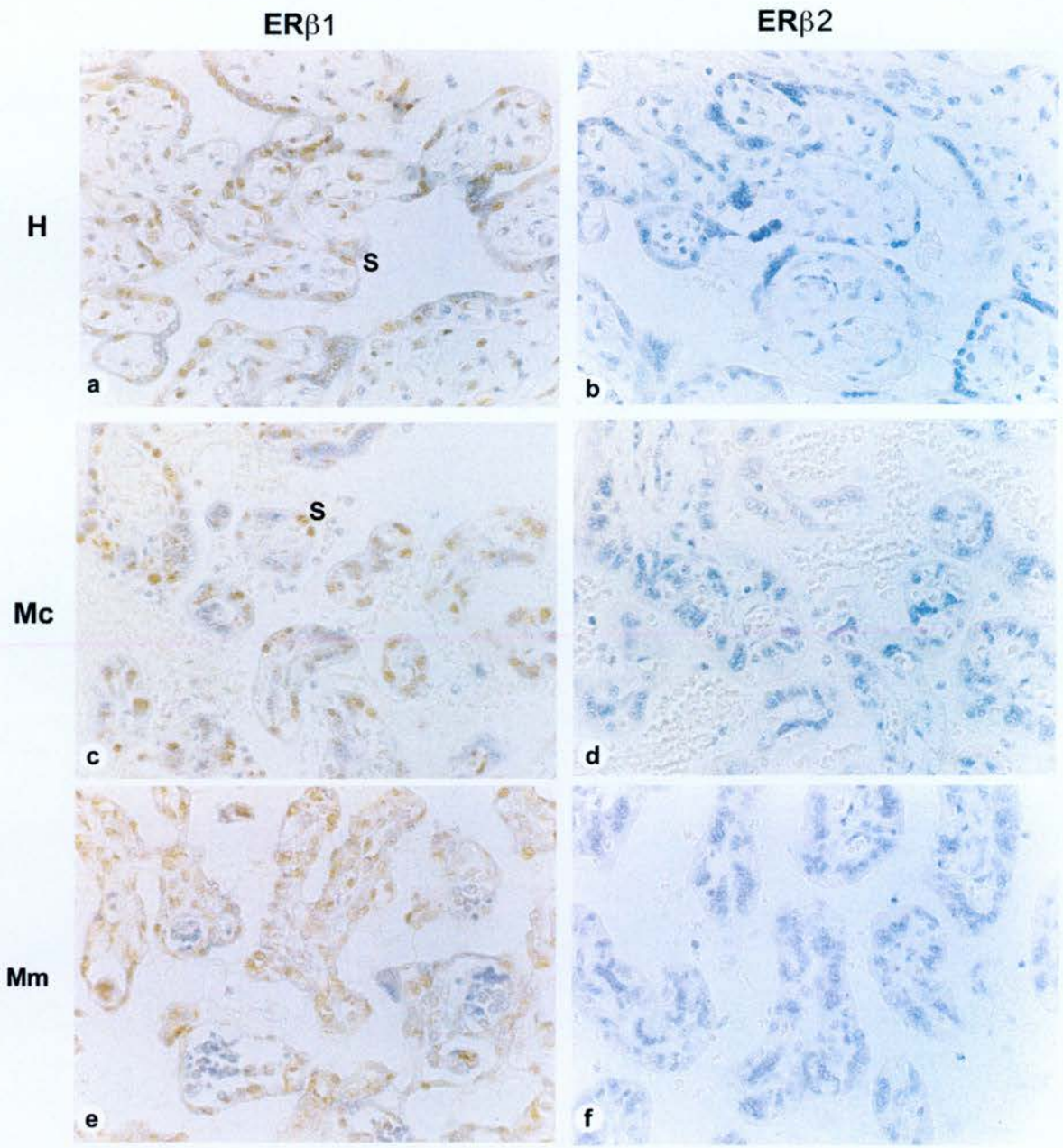


Figure 3.5. Immunohistochemical analysis of ERβ1 and ERβ2 protein expression in the placentas of human, macaque and marmoset. Sections from human (a, b), macaque (c, d) and marmoset (e, f) placenta were stained with the anti-hERβ1 or anti-hERβ2 specific monoclonal antibodies. Positive staining for ERβ1 protein was observed in the nuclei of syncytiotrophoblast (S) cells and in endothelial cells (a, c, e). However when the same placental tissues were stained for the ERβ2 protein none were immunopositive (b, d, f). Magnification is x40.

3.3.2.4 Immunohistochemical Analysis of the Testes

Immunohistochemical evaluation of human, macaque and marmoset testes (*Figure 3.6*) detected intense immunopositive staining for ERβ1 protein in germ cells including pachytene spermatocytes and round spermatids (*Figure 3.6a, c, e*) with less intense reaction in Sertoli cells and spermatogonia. In the human testis ERβ2 protein immuno-stained most intensely in the Sertoli cells and in B type spermatogonia; type A spermatogonia, preleptotene spermatocytes were also immunopositive for ERβ2 protein (*Figure 3b*). There was a low level of immunopositive staining for ERβ2 in human pachytene spermatocytes. Elongated spermatids were immunonegative for both ERβ1 and ERβ2 proteins. Macaque and marmoset testes did not stain immunopositive for ERβ2 with the anti-human ERβ2 antibody.

3.3.3. Immunocytochemcial Analysis of Cell Lines

Immunocytochemical evaluation of the Hek 293 and Hep G2 cell lines confirmed RT-PCR results, with no detectable ERβ1 or ERβ2 protein within the cell nuclei of either cell line (*Figure 3.7a, b, c, d*), although the antibody produced some overall background staining. ERβ1 and ERβ2 proteins were expressed in some cell nuclei following transient transfections with ERβ-containing constructs as positive controls (*Figure 3.7 e, f, g, h*).

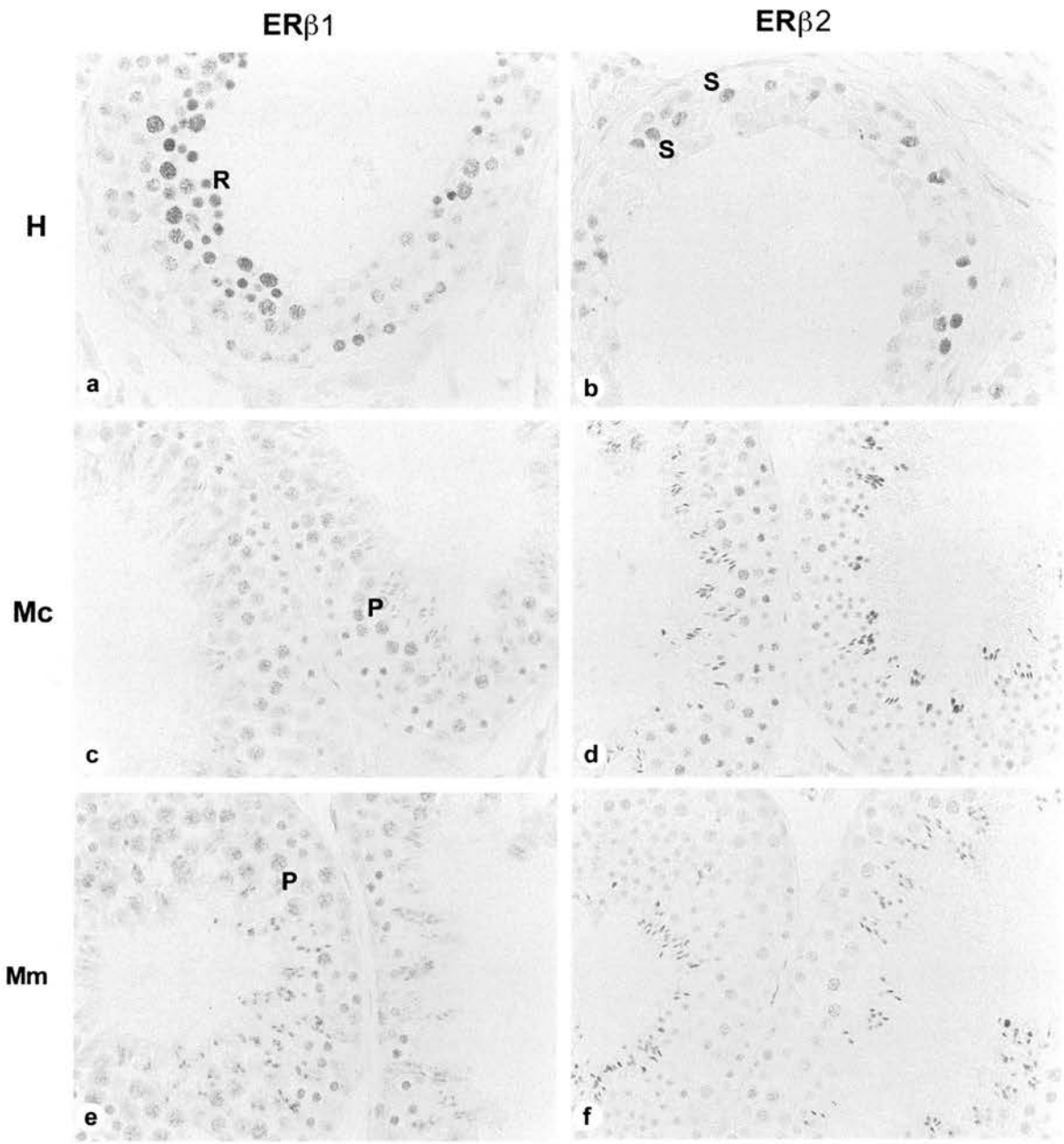


Figure 3.6. Immunohistochemical analysis of ERβ1 and ERβ2 expression in the testis of human, macaque and marmoset. Sections from human (a, b), macaque (c, d) and marmoset (e, f) were stained with the anti-hERβ1 or anti-hERβ2 specific monoclonal antibodies. Immunopositive ERβ1 staining was observed in the nuclei of germ cells including pachytene spermatocytes (P) and round spermatids (R) (a, c, e). However when the testis sections were stained for ERβ2 protein only the human testis was immunopositive (b) with staining detected in the nuclei of Sertoli cells (Sc) and spermatogonia. Testes from macaque (d) and marmoset (f) were immunonegative. Magnification is x40.

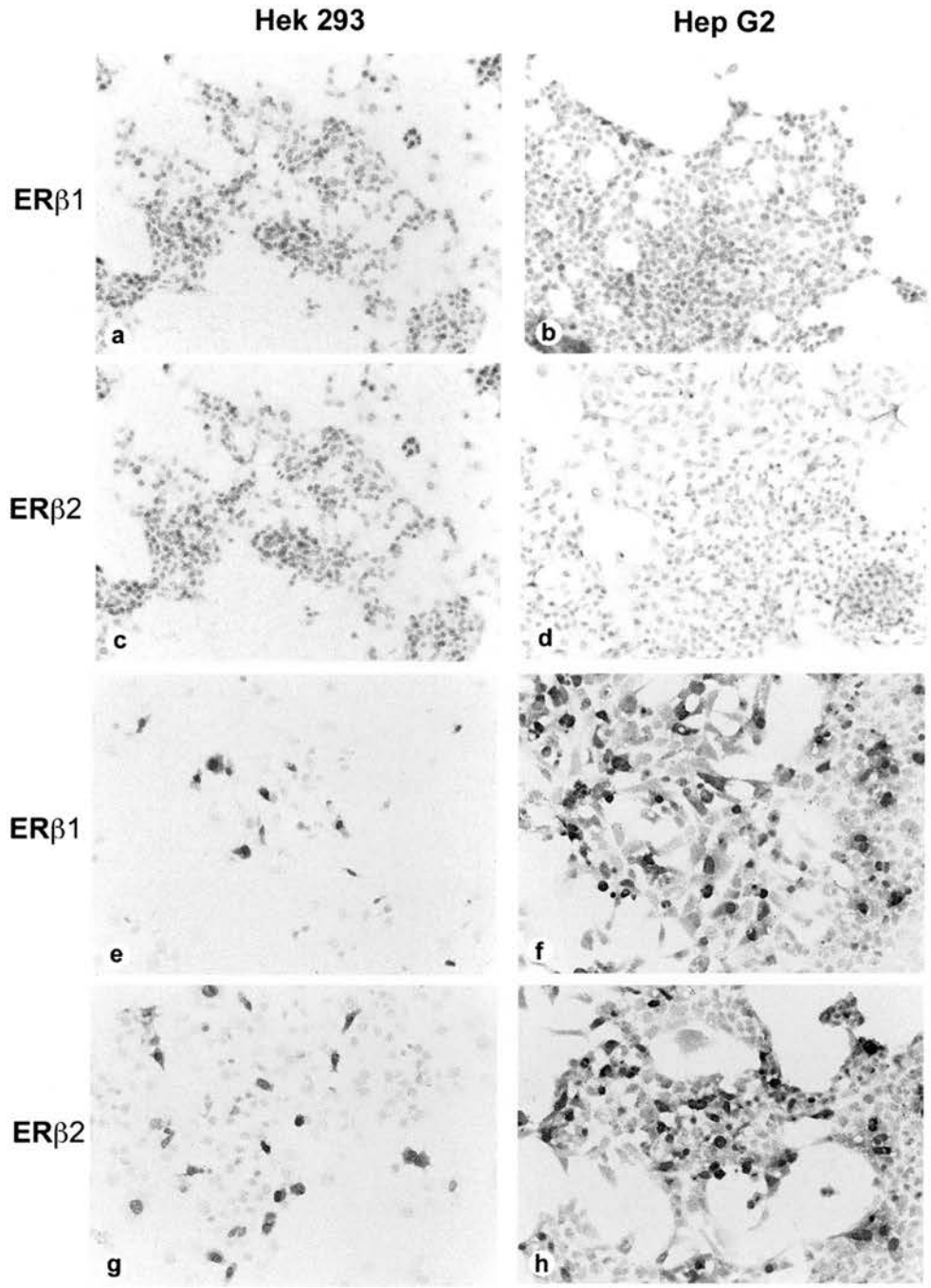


Figure 3.7. Immunocytochemical analysis of ERβ1 and ERβ2 expression in Hek 293 and Hep G2 cell lines. Hek 293 cells (a, c, e, g) and Hep G2 cells (b, d, f, h) were stained with the anti-hERβ1 (a, b, e, f) and anti-hERβ2 (c, d, g, h) specific antibodies. The cells were transiently transfected with human ERβ1 (e, f) or human ERβ2 (g, h) containing expression vectors as positive controls. Untransfected cells showed no immunopositive staining for either ERβ1 or ERβ2 proteins (a, b, c, d), however, following transient transfection some cells showed positive nuclear staining for ERβ1 protein (e, f) and ERβ2 protein (g, h). Magnification is x20.

3.3.4 Sequence Analysis of the Human, Macaque and Marmoset ERβ1 and ERβ2

Cloning and sequencing of full length cDNAs corresponding to macaque and marmoset ERβ1 (*Figure 3.8*) and ERβ2 (*Figure 3.9*) was previously performed by Joseph Gaughan and Graeme Scobie (Accession numbers; macaque ERβ2: AF393815 and marmoset ERβ1: Y09372, ERβ2: AF393816) Alignment of human, marmoset and macaque peptide sequences corresponding to the predicted open reading frames is shown in *Figure 3.8* and *Figure 3.9*.

The ERβ1 proteins showed significant sequence conservation between the three species (490 amino acids), with macaque having 96.6% and marmoset with 93.6% homology to the human. The full length sequences of ERβ2 of human, macaque and marmoset demonstrated differences in the C-terminal region. Marmoset ERβ2 cDNA encoded a full length protein (486 amino acids) with 91.1% homology to human ERβ2. In contrast the macaque ERβ2 cDNA encoded a truncated protein due to the inclusion of a short region of intrinsic DNA containing a stop codon, thus resulting in a protein of 320 amino acids.

Figure 3.8 and *Figure 3.9* show a comparison of the peptide sequences of human, macaque and marmoset ERβ1 and ERβ2. The underlined human peptides are the ones used to raise the antibodies.

Figure 3.8. Comparison of the peptide sequences of human, macaque and marmoset ERβ1.

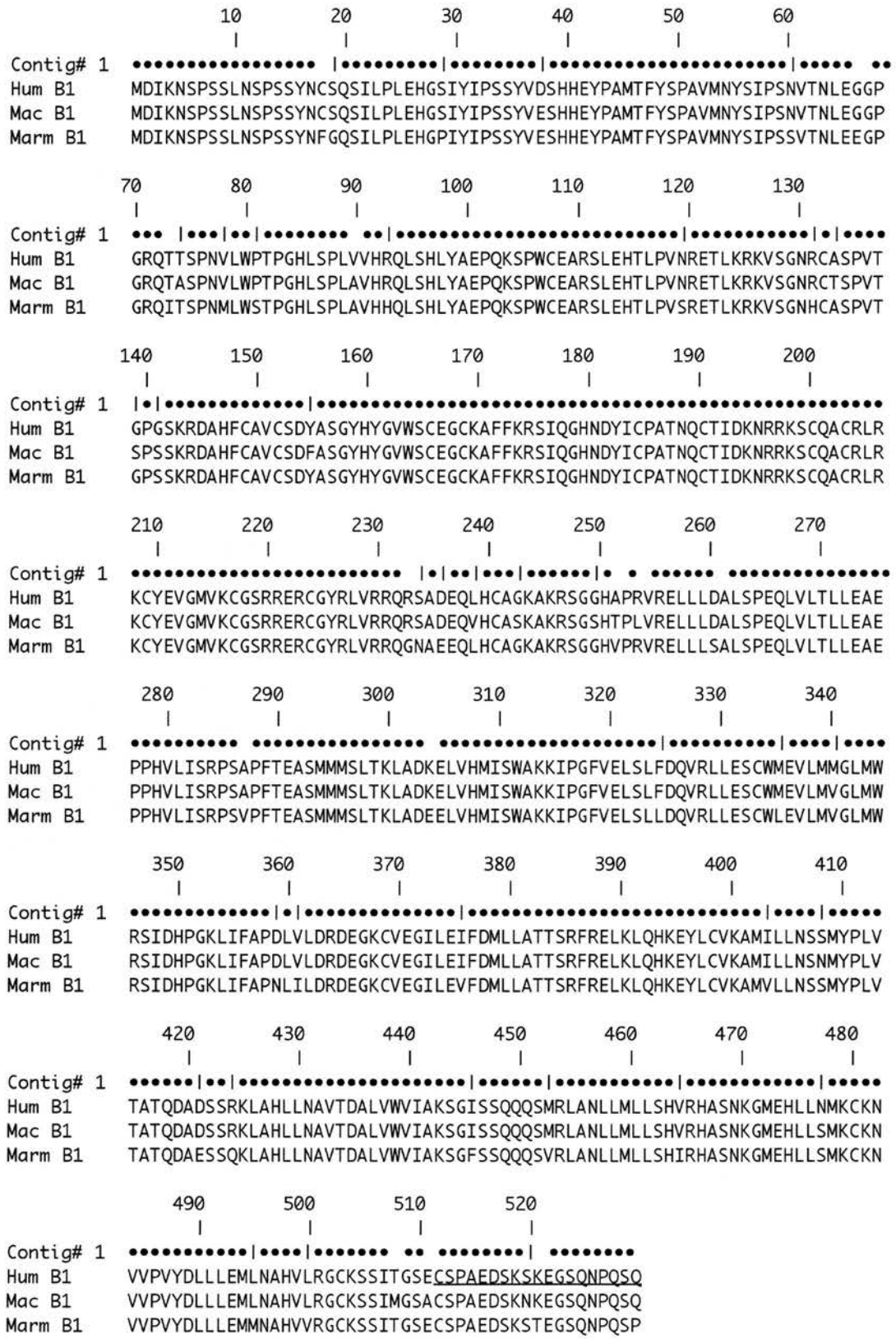
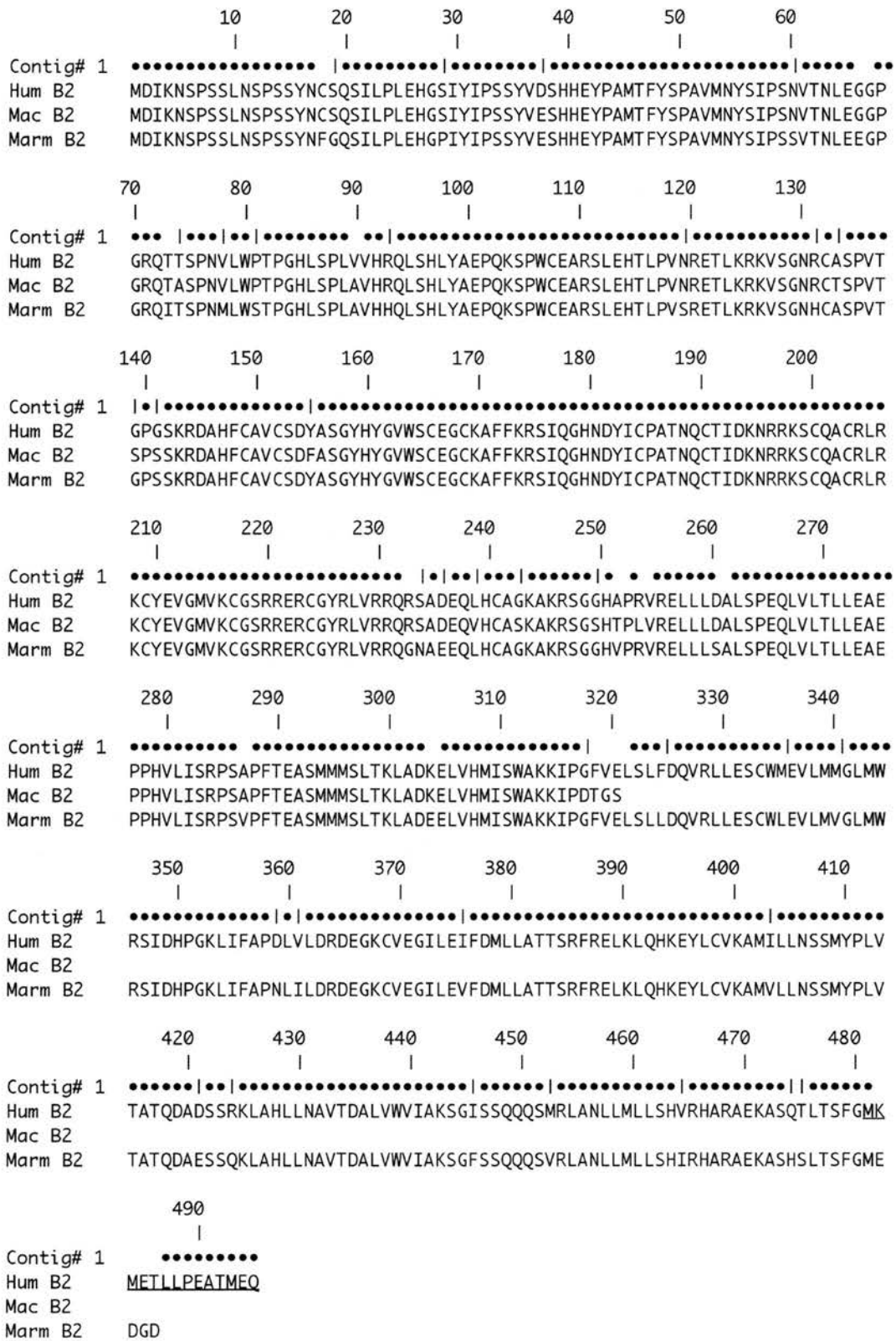


Figure 3.9. Comparison of the peptide sequences of human, macaque and marmoset ERβ2.



3.4 Discussion

Moore *et al* published results in which they investigated the amounts of ERα, full length ERβ (ERβ1) and ERβ variant mRNAs in a wide range of human tissues. Although results were semi-quantitative, they suggested that the amounts of ERβ1 and ERβ2 cDNAs present in different human tissues and cell lines varied, with mRNAs most abundant in the ovary and testis (Moore *et al.*, 1998). Other studies have detected both ERβ1 and ERβ2 mRNAs in samples extracted from human testis, granulosa cells, endometrium, placenta and cells lines (Ogawa *et al.*, 1998c; Saunders *et al.*, 2002a; Scobie *et al.*, 2002). In the present study I found that the levels of ERβ1 and ERβ2 mRNAs in different tissues from human, macaque and marmoset and in the cell lines were variable.

Information regarding the presence of mRNA for ERα, ERβ1 wild type or ERβ2 variant isoforms from RT-PCR is useful. However because a total tissue homogenate extract is used cell specific expression of the receptors cannot be evaluated, therefore immunohistochemical analysis was carried out to identify the precise cells expressing the ERβ1 and ERβ2 proteins. It must also be taken into account that even if there is a message for an ER it does not necessarily indicate that the protein will be expressed.

In this and other studies from our laboratory, the use of fixed, paraffin-embedded tissue combined with mild antigen retrieval resulted in the maintenance of tissue structure and consistent staining results with the antibodies independently generated against specific peptides within ERβ (Atanassova *et al.*, 2000; Critchley *et al.*, 2002; Saunders *et al.*, 2000; Saunders *et al.*, 2001). In all cases immunopositive staining was specific to the nuclei of cells and detected in multiple cell types within the ovary, uterus, placenta and testis, consistent with previous studies (Fisher *et al.*, 1997; Saunders *et al.*, 1998; Saunders *et al.*, 2000).

Using RT-PCR, ERβ1 and ERβ2 mRNAs were detected in Ishikawa, LnCap and Hela cells, but were absent from Hek 293, Hep G2 and Cos 7 cells. Ishikawa cells are transformed human endometrium cells, therefore the detection of ERβ1 and ERβ2 mRNAs is consistent with the ERβ1 and ERβ2 immunopositive staining in human endometrium tissues. Hela cells are derived from cervical cancer cells, LnCap cells are derived from human prostate; both cell lines were found to contain

ERβ1 and ERβ2 mRNA. ERβ mRNAs were not detected in Hek 293 (human kidney), Hep G2 (human liver) or Cos 7 (African Green Monkey Kidney cells). The absence of ERβ from the Cos 7 cells maybe due to species specific primers used to amplify the cDNA pools, or it may correspond to human and marmoset kidney cDNA pools not containing mRNA for ERβ1 or ERβ2. Consistent with RT-PCR, immunocytochemical analysis of the Hek 293 and Hep G2 cells did not detect ERβ1 or ERβ2 protein expression, however upon transfection with ERβ1 or ERβ2 containing constructs some nuclei stained immunopositive.

ERβ2 variant mRNA was detected in the testis, ovary and endometrium of human, macaque and marmoset using RT-PCR, however the anti-human ERβ2 antibody used in these immunohistochemical studies did not detect ERβ2 protein expression in the macaque or marmoset. Sequence comparisons between the ER proteins encoded by the human, macaque and marmoset cDNAs revealed that the peptide sequence (P8) in the C-terminus used to raise the monoclonal antibody was not present in the primate ERβ2 sequences and this is consistent with the failure of the anti-human ERβ2 antibody to detect primate ERβ2 proteins.

Previous studies using a polyclonal anti-ERβ antibody detected ERβ immunopositive cells in all three species (data not shown and Saunders *et al.*, 2000; Saunders *et al.*, 2001). This antibody was raised against a conserved peptide between the species, within the hinge domain of ERβ (P4) (*Figure 3.1*) and therefore can detect both ERβ1 and ERβ2 in the human, macaque and marmoset. Comparisons between the pattern of expression of ERβ determined using the polyclonal antibody and those obtained using the anti-human ERβ1 monoclonal antibody provide some supporting evidence that ERβ variant proteins are expressed in primate tissues. For example, examination of the pattern of ERβ expression in marmoset and macaque testis illustrated in Saunders *et al.*, (2001), reveals that Sertoli cells are immunopositive. In the present study and a previous one (Saunders *et al.*, 2002a) ERβ1 immunoreactivity in Sertoli cell nuclei has been shown to be faint. In human, ERβ2 is strongly expressed in Sertoli cell nuclei and it is therefore possible that some reactivity seen in the primate Sertoli cell stained with the anti-ERβ hinge antibody may be attributed to expression of macaque or marmoset ERβ2 proteins in these cells.

In the ovary ERβ1 protein expression was intense in the granulosa cells of all three species, however ERβ2 protein was not detected in the macaque or marmoset although ERβ2 mRNA was detected in ovary cDNA pools. ERβ1 protein was also detected in thecal cells, the corpus luteum and the surface epithelium of the ovaries (data not shown). Previous studies using the anti-ERβ hinge domain antibody have detected ERβ protein in the nuclei of granulosa cells in ovaries taken from human and marmoset during the early, mid and late stages of follicular phase and in some cells of the corpus luteum (Saunders *et al.*, 2000; Saunders, 2002). In those studies, immunopositive cells were detected in follicles both before and after formation of an antrum, but absent from cells within an atretic follicle. Therefore my results and those previously published (Saunders *et al.*, 2000) and reviewed in Saunders (2002), demonstrate that ERβ protein is expressed in multiple cell types within the human and primate ovary. However, the issue of whether ERβ2 is present in germ cells of primates cannot be resolved as ERβ1 present, therefore polyclonal data could be due to either ERβ1 or ERβ2 or both.

Investigators have generally reported that the amounts of ERβ mRNA in the endometrium are lower than those of ERα (Critchley *et al.*, 2002; Matsuzaki *et al.*, 2000) and that ERβ mRNA levels are lower in the secretory phase compared to the proliferative phase. Quantitative Taqman PCRs have been performed to detect the levels of ERβ1 and ERβ2 in the human endometrium (Critchley *et al.*, 2002). The levels of both mRNAs were found to be significantly increased in samples obtained during the late secretory phase of the cycle compared to other stages. The overall pattern of both ERβ1 and ERβ2 variant mRNAs paralleled each other with the lowest levels detected during the proliferative/early secretory stages. In the results shown here, mRNA for ERβ1 and ERβ2 was present in the endometrium from all three species analysed.

Immunohistochemistry has previously demonstrated that ERα protein is expressed in the glandular and stromal nuclei in the endometrium (reviewed in Saunders, 2002) and that there is a difference in ERα and ERβ expression in the vascular endothelium and the perivascular cells surrounding the endometrial blood vessels, with only ERβ being detected in the endothelial cell population, although both ERα and ERβ are present in perivascular cells (Critchley *et al.*, 2001; Saunders, 2002).

ERβ protein expression within the vascular endothelium is reported to increase in the mid-late secretory phase (days 24-26) (Lecce *et al.*, 2001).

Immunohistochemistry to determine the spatial and temporal expression of ERβ1 and ERβ2 proteins in human uterine tissues has detected expression of both isoforms within the cells lining the glands and in some stromal cells within the functional and basal layers (Critchley *et al.*, 2002). The functional layer had the most intense staining for ERβ1 in the glandular epithelium and this was especially marked in the mid secretory phase, whereas the ERβ2 protein was less intense in the mid secretory phase. Levels of ERβ1 expression within the basal layer were generally more intense than those of ERβ2, especially within the glandular epithelium. Levels of ERβ1 and ERβ2 protein did not appear to vary across the cycle (Critchley *et al.*, 2002).

The results in the present study were in agreement with a previous study on human endometrium (Critchley *et al.*, 2002). The macaque and marmoset endometrial tissues were also found to express the ERβ1 protein in the endometrial cells, in the stromal cells and those lining the glands. As expected, ERβ2 protein was not detected in the primate endometrium using the antibody raised to human ERβ2.

Therefore it can be concluded from previous studies and the work presented here that both ERβ1 and ERβ2 mRNAs and proteins are expressed within the endometrium but at lower levels than ERα. The levels of expression of ERβ1 and ERβ2 throughout the cycle do not change as dynamically as does ERα protein expression. The immuno-staining for ERβ1 is more intense compared to ERβ2, but both receptors are present in cell nuclei in the functional and basal layers. However, it must be taken into account that immunohistochemical analysis is not quantitative and therefore differences in the intensity of ERβ1 or ERβ2 protein expression may not be accurate. Therefore quantitative RT-PCR is required to deduce the changes in ERβ1 and ERβ2 mRNA levels throughout the cycle, although to determine relative levels in stroma and glands this would need to be performed after laser capture microscopy (Gaskell *et al.*, 2003).

It has previously been shown that ERα protein is expressed in human decidual cells of the placenta from early pregnancy (Wu *et al.*, 1993). However, in the third trimester ERα protein was not detected in the decidual tissue (Noci *et al.*, 1994;

Saunders, 2002). ERβ protein has been detected in the decidualised stromal cells and the syncytiotrophoblast in the second and third trimester human placenta (Henderson *et al.*, 2003; Lecce *et al.*, 2001; Saunders, 2002). The results shown here demonstrate for the first time that ERβ1 protein is also expressed in placental tissue from macaque and marmoset. ERβ1 protein is expressed within the nuclei of perivascular cells surrounding the blood vessels and the syncytiotrophoblast in human, macaque and marmoset placenta. ERβ2 protein was not detected in human, macaque or marmoset placental tissues. These results were consistent with the failure to detect ERβ2 mRNA following RT-PCR analysis of RNA from human and marmoset placenta.

Results in this study and others (Makinen *et al.*, 2001; Scobie *et al.*, 2002) using RT-PCR analysis have demonstrated that ERβ1 and ERβ2 mRNAs are both expressed within the testes of humans, as well as macaques and marmosets. However, upon closer examination immunohistochemical evaluation in this and previous studies has shown that the localisation of ERβ1 and ERβ2 is very different. ERβ1 protein is expressed in the nuclei of pachytene spermatocytes and round spermatids, faint levels of expression are detected in Sertoli cells, spermatogonia, preleptotene, leptotene, zygotene and diplotene spermatocytes (Saunders *et al.*, 2002a; Saunders, 2003). Highest levels of ERβ2 protein were found to be present in human Sertoli cells and spermatogonia with low or variable expression in preleptotene, pachytene and diplotene spermatocytes, and most interstitial cells expressed more ERβ2 than ERβ1 (Saunders *et al.*, 2002a). ERβ2 protein was not detected in macaque or marmoset testes, however, ERβ2 mRNA was detected in the macaque and marmoset tissue. Immunohistochemical analysis using the polyclonal antibody raised against the conserved hinge region (P4) has demonstrated the presence of immunopositive cells within the testis in the primates, which do not stain immunopositive for ERβ1 (data not shown and Saunders *et al.*, 2001).

ERα protein and mRNA is reported to be absent from testis of humans and primates (Makinen *et al.*, 2001; Saunders *et al.*, 2001), but levels of oestrogen are higher in the male testis than in the rest of the circulation (Hess *et al.*, 1997), therefore ERβ is presumed to function as the target oestrogen receptor. However, as ERβ2 is present but differentially expressed compared to ERβ1, the ERβ2 variant may have a specific role within the testis which still needs to be elucidated.

Co-localisation studies of both ERβ1 and ERβ2 on the same human sections would be valuable, however this has not been possible as both antibodies were raised in mouse and although they were different monoclonal IgG subtypes, secondary antibody specificity was poor. Localisation of the macaque and marmoset ERβ2 with a specific ERβ2 antibody raised to their respective ERβ2 peptide sequences instead of the human ERβ2 would be extremely useful. It would then be possible to observe the specific localisation of ERβ2 rather than assuming it's localisation by comparing staining using the anti-ERβ hinge antibody, which detects both ERβ1 and ERβ2 variant proteins, with that seen with the anti-ERβ1 specific monoclonal antibody.

Patterns of expression in different tissues reveal potential complexity in response of individual cells to oestrogens. For example, in the human ovary ERβ1 protein is expressed in all granulosa cells and it is assumed that ERβ2 protein may also be expressed in all granulosa cells. In the endometrium, ERα protein levels alter throughout the cycle, ERβ1 is present in vascular endothelial cells, whereas ERβ2 protein is not detected in endothelial cells. In the placenta, only ERβ1 protein has been detected. In the human and primate testes, ERα is not detected, however germ cells stain immunopositive for ERβ1 protein, and Sertoli cells stain immunopositive for ERβ2 protein. Therefore the differential distribution of the oestrogen receptors may have an affect on the pattern of gene expression induced by oestrogenic ligands the target tissues are exposed to.

It has been shown ERα and ERβ are able to form heterodimers (ERα/ERβ1) upon ligand binding as well as homodimers (ERα/ERα, ERβ1/ERβ1) (Cowley *et al.*, 1997). In addition Moore *et al* used *in vitro* gel shifts assays to show that ERβ2 can form heterodimers with ERα or ERβ1 as well as homodimers (Moore *et al.*, 1998). In the following chapters experiments defined to evaluate the impact of steroid ligands on subcellular localisation of ERβ1 and ERβ2 proteins and reporter gene activation are described.

Chapter 4

Investigations on the Functional Competence of ERα, ERβ1 and ERβ2 Using FP-tagged Proteins

4.1 Introduction

In Chapter 3 the expression of ERβ1 wild type and ERβ2 variant were investigated. Evidence was obtained demonstrating the differential expression of ERβ1 and ERβ2 mRNA and protein in human and primate tissues. In several target tissues such as the brain, ovary and mammary gland previous published data has shown that ERα and ERβ are co-expressed (Saunders *et al.*, 2000; Saunders *et al.*, 2002b; Wang *et al.*, 2003), however in other tissues such as the human and primate testes ERα is absent and ERβ1 and ERβ2 are abundant (Saunders *et al.*, 2002a; Scobie *et al.*, 2002). Previous published work has also demonstrated that ERα and ERβ can heterodimerise upon ligand binding (Cowley *et al.*, 1997; Pettersson *et al.*, 1997) and that the heterodimers are able to bind to an ERE with an affinity similar to that of the ERα homodimer and greater than that of the ERβ homodimer (Pettersson *et al.*, 1997). Understanding the distribution of the ERs is important as it has been demonstrated that ligands bind to the ER isoforms with differing affinities and are able to influence gene transcription to different extents (Hall and Korach, 2002; Hall *et al.*, 2002).

4.1.1 Subcellular Oestrogen Receptor Distribution

In cells ERs have been detected in the nucleus by immunocytochemistry (Chapter 3 and Htun *et al.*, 1996; Stenoien *et al.*, 2000) and hormone binding assays (Beato and Sanchez-Pacheco, 1996). Receptor movement in the nucleus is reported to be highly dynamic with the receptors undergoing constant exchange between genomic regulatory elements, multiprotein complexes with other transcription factors and subnuclear structures which have not yet been fully identified (Hager *et al.*, 2000). The oestrogen receptor has been demonstrated to move from a chaperone complex (8s) to a dissociated form (4s) in a ligand dependent fashion, but it is constantly present within the nucleus (Hager *et al.*, 2000). Hormone binding leads to a conformational change in the receptor that results in the dissociation of the receptor

from the chaperone and ultimately in the binding of the receptor as a dimer to cognate sites in steroid responsive genes (Beato and Sanchez-Pacheco, 1996; Htun *et al.*, 1999; Tsai and O'Malley, 1994).

In the absence of ligand, ERα tagged to a fluorescent protein (FP) is distributed in a reticular (network) pattern evenly distributed throughout the nucleus, therefore suggesting that the majority of the unligand bound receptor is not freely diffusing in the nucleus but is associated with nuclear meshwork (Htun *et al.*, 1999). Upon addition of ligand there is a dramatic redistribution of the receptors to give a punctate or focal pattern. These discrete focal clusters of hERα are associated with the nuclear matrix and co-factors within the nucleus (Htun *et al.*, 1999). The two distinct nuclear localisation patterns provided the first visual evidence of the changes in receptor activity by hormones and reflect the loose and tighter binding nuclear forms (Greene and Press, 1986).

ERs control the expression of specific genes by binding to regulatory DNA sequences (EREs) located at the promoter or enhancer regions of the genes, however it has been shown that ERα foci are not mainly localised at the sites of nascent mRNA transcription (Matsuda *et al.*, 2002). This ligand-dependent intranuclear reorganisation of the oestrogen receptor into accumulation centres involves more complex events than simple recognition of specific chromosomal DNA binding. Therefore a highly organised and dynamic subnuclear environment provides the framework for receptor function (Stenoien *et al.*, 2001b).

Ligand binding, dimerisation, DNA binding, binding with co-factors and binding with the nuclear matrix are all required for ERs to form discrete foci (Matsuda *et al.*, 2002). The C-terminal region of the ER (E/F domain) plays an important role in the receptor function because this region is essential for ligand binding and ligand dependent transcriptional activation through binding with co-activators and dimerisation. In recent studies deletion of the N-terminal region encoding the AF-1 domain required for co-factor regulation prevented discrete clusters forming (Matsuda *et al.*, 2002). Co-expression of a mutated ERα-tagged to an FP with an intact FP-ERα restored the capacity to form discrete clusters, therefore indicating that one AF-1 region within the receptor dimer was sufficient for the cluster formation (Matsuda *et al.*, 2002). A single DNA binding (C) domain was also found to be sufficient for cluster formation with ER dimers (Matsuda *et al.*, 2002).

However, truncated forms of the hERα that lack the E/F and therefore the AF-2 domain do not have the ability to form discrete clusters shown using different FP constructs (Matsuda *et al.*, 2002), therefore suggesting two functional AF-2 domains within the ER dimer are required for transcriptional activity (Matsuda *et al.*, 2002; Tremblay *et al.*, 1999a). Weatherman *et al* also demonstrated that ER dimer interactions are wholly dependent upon AF-2 in hERα (Weatherman *et al.*, 2002).

4.1.2 Ligands

This chapter examines the distribution of the ER isoforms tagged to a fluorescent protein and the alteration of the FP-ER distribution upon exposure to selected ligands. Previous studies have concentrated on FP-ERα and its redistribution with 17β-oestradiol (E_2) (Htun *et al.*, 1996; Stenoien *et al.*, 2001b), tamoxifen (Htun *et al.*, 1999) and the antagonist ICI 182,780 (Htun *et al.*, 1996). Several studies have demonstrated that ERα and ERβ1 exhibit different affinities for ligands such as 3βAdiol and genistein, (Harris *et al.*, 2002a; Weihua *et al.*, 2002), as well as for novel ligands which act selectively as agonists or antagonists for ERα and ERβ1 (Sun *et al.*, 1999). Therefore in this study different ligands (E_2, 3βAdiol, genistein, DPN™ and PPT™) were analysed for their abilities to activate the ERs, assessed by their ability to redistribute the fluorescently tagged receptor within an individual live cell.

Oestradiol is the classical ligand for the oestrogen receptor and the two receptor subtypes have a similar affinity for E_2 despite sequence differences within their LBDs (Kuiper *et al.*, 1996; Ogawa *et al.*, 1998b). The metabolite of 5α-dihydrosterone (DHT); 5α-androstane-3β,17βAdiol (3βAdiol) has been shown to be a potent hormone (Weihua *et al.*, 2002). The oestrogenic activity of 3βAdiol has been documented previously and it has been found to displace E_2 (Hackenberg *et al.*, 1993; Ho and Ofner, 1986). It has previously been reported that ERβ1's affinity for 3βAdiol is greater than ERα's and compared to E_2, it's affinity is 10-fold lower for ERβ1 and 30-fold lower for ERα (Weihua *et al.*, 2002).

It has been demonstrated that certain phytoestrogens, such as the isoflaviniod genistein, have the ability to activate transcription of ERβ1 to a greater extent (approximately 30-fold) than ERα (An *et al.*, 2001; Barkhem *et al.*, 1998; Harris *et al.*, 2002a; Kuiper *et al.*, 1997). Genistein alters the helix 12 conformation in ERα in

an agonistic manner, however with ERβ1 the alteration in helix 12 conformation is not the same as when the receptor is bound to agonists, but instead is only partially altered (Liu *et al.*, 2003). Therefore, the difference in binding affinity of genistein for ERβ1 does not relate directly to the transcriptional activation because genistein is over 1000-fold more potent at triggering transcriptional activity with ERβ1 compared with ERα (An *et al.*, 2001).

Attention has recently been focused on developing novel ligands that are capable of activating either receptor isoforms in a specific manner. Two such commercial ligands have been used in this study; Propyl pyrazole triol (PPT™) and 2,3-bis(4-hydroxyphenyl)propionitrile (DPN™). PPT™ is a potent agonist for human ERα but is inactive on hERβ1, becoming an hERβ1 agonist only at very high concentrations (Kraichely *et al.*, 2000; Stauffer *et al.*, 2000). Following binding it has the ability to stabilise hERα to the same extent as E_2.

Meyers *et al* has recently developed a non-steroidal ERβ1-selective ligand; diarylpropionitrile (DPN™). DPN™ acts as an agonist on both the hERα and hERβ1 subtypes, but has a 70-fold higher relative binding affinity and a 170-fold relative potency in transcription assays with hERβ1 than with hERα (Meyers *et al.*, 2001). This is a substantially higher level of hERβ1 affinity and potency selectivity than that of the partial ERβ1 agonist genistein (Meyers *et al.*, 2001).

4.1.3 The Effects of Co-transfections with ERα, ERβ1 and ERβ2 Variant

As discussed in Chapter 3, the localisation of oestrogen receptors within tissues is very diverse. In many tissues different ER isoforms are expressed in different cells, however co-expression of the isoforms can occur within one cell. It has been documented that ER isoforms not only form homodimers upon ligand binding, but also form heterodimers for example ERα/ERβ1 (Cowley *et al.*, 1997; Ogawa *et al.*, 1998b; Pettersson *et al.*, 1997). These studies have looked at the binding abilities of the receptors and analysed the effect of heterodimerisation on transcriptional activity, however there has only been one previous publication demonstrating dimerisation between rat ERα and ERβ in live cells using FP-tagged constructs (Matsuda *et al.*, 2002). Studies using FP-ERβ2 have not been reported previously.

4.1.4 Fluorescent Proteins

Since the development of vectors which allow preparation of proteins linked to fluorescent proteins (FP), it is now possible to use real time monitoring to observe a tagged protein's distribution within the live cell. The properties of FPs make them useful as molecular reporters to monitor patterns of protein localisation, gene expression and intracellular protein trafficking in living cells (Ogawa *et al.*, 1995), therefore avoiding potential artefacts due to cell fixation and permablisation (Tavare *et al.*, 2001).

The green fluorescent protein (GFP) was discovered (Shimomura, 1962) as a companion protein to aequorin, the chemiluminescent protein from *Aequorea* jellyfish. GFPs are found in a variety of bioluminescent organisms ranging from the jellyfish *Aequorea Victoria* to the sea anemone relative *Discosoma* (Tsien, 1998). Wild type GFPs possess the property of accepting energy by radiation-free energy transfer process from an excited state blue fluorescent protein during the aequorin bioluminescence reaction, with a maximum peak of absorbance at 395 nm and a minor peak at 470 nm. Several chromophore variants have been placed into plasmid vectors by Clontech Laboratories including the green fluorescent protein (EGFP) from the *Aequorea victoria* jellyfish and the red fluorescent protein (DsRed) derived from the *Discosoma sp.* The availability of different coloured FPs allows two or more proteins to be tagged with these distinct spectral variants, thus enabling them to be localised in cells simultaneously.

The fluorescent tag can be positioned at the N- or the C-terminus of the test protein. Hager *et al* placed a GFP tag at the amino terminus of human ERα and observed that the receptor was expressed as a stable protein and that it retained transactivational properties similar to those of the untagged receptor (Hager *et al.*, 2000). This was supported by Price *et al* who found that the GFP tag positioned at the N-terminus of the ER, does not alter the binding characteristics of ERα or ERβ1, nor the localisation of the receptors (Price *et al.*, 2001).

4.1.5 Aims

The aim of the experiments described in this chapter was to assess the subcellular distribution of oestrogen receptors (ERα, ERβ1 and ERβ2) using fluorescently tagged constructs. The redistribution of the fluorescently tagged ERs in the presence and absence of different ligands was studied to evaluate the activation potential of the ligands. Two different fluorescent tags were used during the study to be able to visualise the receptor isoforms co-transfected together and to assess whether they could co-localise following addition of ligand with the formation of heterodimers.

4.2 Methods

4.2.1 Generation of ERβ Fluorescent Constructs

To be able to observe the behaviour of human ERβ1 or ERβ2 proteins expressed in living cells, full length cDNAs were cloned into fluorescent protein vectors (EGFP and DsRed) purchased from Clontech. These vectors were chosen so the human ER cDNAs were inserted into the vector in frame with the 3' end of the EGFP or DsRed. The resulting protein was therefore tagged with an N-terminal EGFP or DsRed protein. The position of the tag was chosen to avoid disturbing the ligand binding or AF-2 domains of the steroid receptors.

4.2.1.1 Preparation of the Fluorescent Plasmid Vectors

EGFP- and DsRed-containing plasmid vectors (Clontech) were transformed into XLI Blue cells, plated out onto kanamycin-containing plates and propagated in LB with kanamycin (section 2.8). A CsCl preparation was performed to ensure the vectors were pure prior to cloning (section 2.9.2.3).

4.2.1.2 Preparation of the ER cDNAs and the Florescent Vectors

Human ERβ1 and hERβ2 were amplified up from pUni and pRSET vectors (respectively) by PCR with AGS Gold Taq as discussed in section 2.5 and 2.7. The PCR products were purified by elution through a High Pure Purification column (section 2.7.2).

4.2.1.3 Cloning of ERβ1 and ERβ2 into the Fluorescent Vectors

The hERβ1 and hERβ2 cDNAs and the fluorescent vectors were digested with HindIII and BamHI (section 2.8.2) and the cDNAs ligated into the vectors (section 2.8.3). Once the constructs had been generated the plasmids were screened by restriction digestion and sequenced to ensure the cDNAs had been cloned correctly (section 2.8.5). The plasmids containing the correct constructs were prepared by the CsCl method prior to transient transfection studies being performed (section 2.9.2.3).

4.2.2 Human ERα -pDsRedN1

Human ERα cloned into the pDsRed-N1 vector was a gift from F. Schaufele (Department of Medicine, University of California, San Francisco, USA. (Weatherman *et al.*, 2002)). The plasmid was propagated and purified using the CsCl method (section 2.9.2.3).

4.2.3 Transient Transfections for Confocal Microscopy

Transfections were performed as described in section 2.10.2.5, Hek 293 or Hep G2 cells were plated onto 35mm glass bottom microwell dishes (Plastik® Corporation). For each dish used, 2μg of DNA was transfected into the cells using JetPEI (section 2.10.2.2). In the co-transfection experiments a total of 2μg ER (1μg of each ER) was used. After 48 hours the media was removed and the cells washed in PBS at room temperature. To maintain the cells during analysis, 1ml PBS with 25mM Hepes buffer (Sigma) was added to the dish. The ligand used was diluted to 10^{-8}M in PBS/Hepes and added to the dish that was already in place on the warm plate (37°C) on the confocal microscope (LSM510, Zeiss). Images were captured at 10 minute intervals for 1-3 hours and exported into Photoshop 7.0 (Adobe).

The confocal pictures obtained correspond to the FP colours and the yellow regions represent co-localised ERs tagged with the green and red FPs in the co-transfection studies. The intensity profiles were obtained using the confocal software; a line was drawn through the length of a selected nuclear image and the profile generated.

4.2.4 Transient Transfections for Western Analysis

Cells were plated out into 60mm dishes (Cellstar®) as described in section 2.10.2.6 and transfected with 6μg of plasmid DNA. The cells were incubated for 48 hours prior to being harvested using the crude whole cell protein extraction method (section 2.10.2.6.1.3).

4.2.5 Western Analysis

The protein samples obtained from the ERβ-EGFP transient transfections were separated on a Western gel as described in section 2.10.2.6.3. The anti-EGFP mouse monoclonal antibody was purchased from Clontech (Catalogue Number; 8367-2), used at a 1:500 dilution and was incubated with the membrane overnight at 4°C. The secondary antibody used was a mouse anti- rabbit IgG (Diagnostics Scotland) used at 1:5000, incubated with the membrane in TBST at room temperature for 2 hours. The Western membrane was developed with ECL Plus Detection Kit (Amersham Life Sciences) as described in section 2.10.2.6.3.4.

4.3 Results

4.3.1. Western Showing ERβ Protein Present

The Western blot (*Figure 4.1*) shows that the transiently transfected FP-tagged ERs were present in the transfected cells, giving a band at approximately 86.2 kDa for ERβ1 and at 82.5kDa for ERβ2. Results are consistent with the expected sizes of ERβ1 protein (59.2 kDa) and ERβ2 (55.5 kDa) tagged with the EGFP (27 kDa). The protein was detected within the "supernatent" (cytosol and nuclear protein) extract, whereas the "pellet" (residual nuclear debris) did not contain any protein.

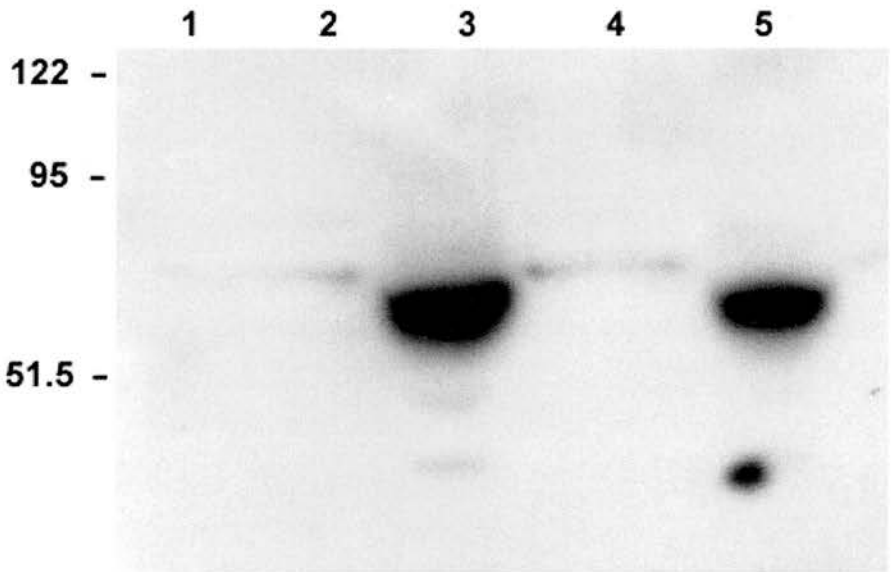


Figure 4.1. Western analysis of extracts prepared from cells transfected with EGFP-tagged ERβ. Samples run were (1) Blank, (2) ERβ1-EGFP; pellet, (3) ERβ1-EGFP; supernatant, (4) ERβ2-EGFP; pellet, (5) ERβ2-EGFP; supernatant. The proteins were detected using an anti-EGFP antibody (Clonetch). The molecular weight marker was run alongside the samples to determine the band size.

4.3.2 Treatment of Cells with E_2

Following transient transfection ERα-DsRed was localised to the nucleus in both Hek 293 and Hep G2 cells (*Figures 4.2a & 4.3a*). In the absence of E_2 the protein was diffusely distributed with a slight speckled "focal" arrangement (*Figures 4.2a* & *Figure 4.3a* t = 0). Addition of the ligand resulted in relocation of the ERα-DsRed to form bright regions (foci) representing a high intensity of the ERα, within 10 minutes. The number and brightness of the foci increased and appeared to be maximal at 30 minutes, with a decrease in the diffusely distributed receptor. The effect of the ligand lasted until 60 minutes, after which the foci started to disperse and the receptor began to relocate into a diffuse pattern again. The intensity profiles (*Figures 4.2b* & *4.3b*) illustrates the focal pattern of the receptor, showing the greatest intensity peaks where foci are brightest (arrows). The peaks of the intensities and their frequency as well as the speckled background varied between cells and between the two cell lines. In Hep G2 cells the intensity profiles demonstrate peaks in intensity to a greater extent and the foci were more discrete than in Hek 293 cells.

In the absence of E_2 (t = 0), ERβ1-EGFP transiently transfected into Hek 293 and Hep G2 cells (*Figures 4.4a* & 4.5*a*) showed a diffuse pattern. Typically a couple of discrete foci were observed in both cell lines, which developed into brighter foci upon addition of 10^{-8}M E_2. Redistribution of ERβ1-EGFP into bright discrete foci was observed after 20 minutes incubation with 10^{-8}M E_2. Thereafter the number of foci increased and the diffuse ERβ1-EGFP reduced. At 60 minutes the greatest number and brightness of discrete foci was observed with the least diffuse ERβ-EGFP present. From the intensity profiles it can be deduced that not all the cell nuclei have the same intensity and there is a difference in the number and appearance of foci.

It was noted that ERα and ERβ1 tagged to the FP had a different focal appearance. ERα was observed to form regions of bright foci upon addition of ligand whereas ERβ1 formed more discrete foci in both of the cell lines used. Results are summarised at the end of the results in *Tables 4.1 & 4.2*.

ERβ2-EGFP transiently transfected into Hek 293 and Hep G2 cells (*Figures 4.6a & 4.7a*) adopted a diffuse pattern in absence of ligand (t = 0) and remained in a diffuse distribution after incubation with E_2 for one hour irrespective of the cell line used.

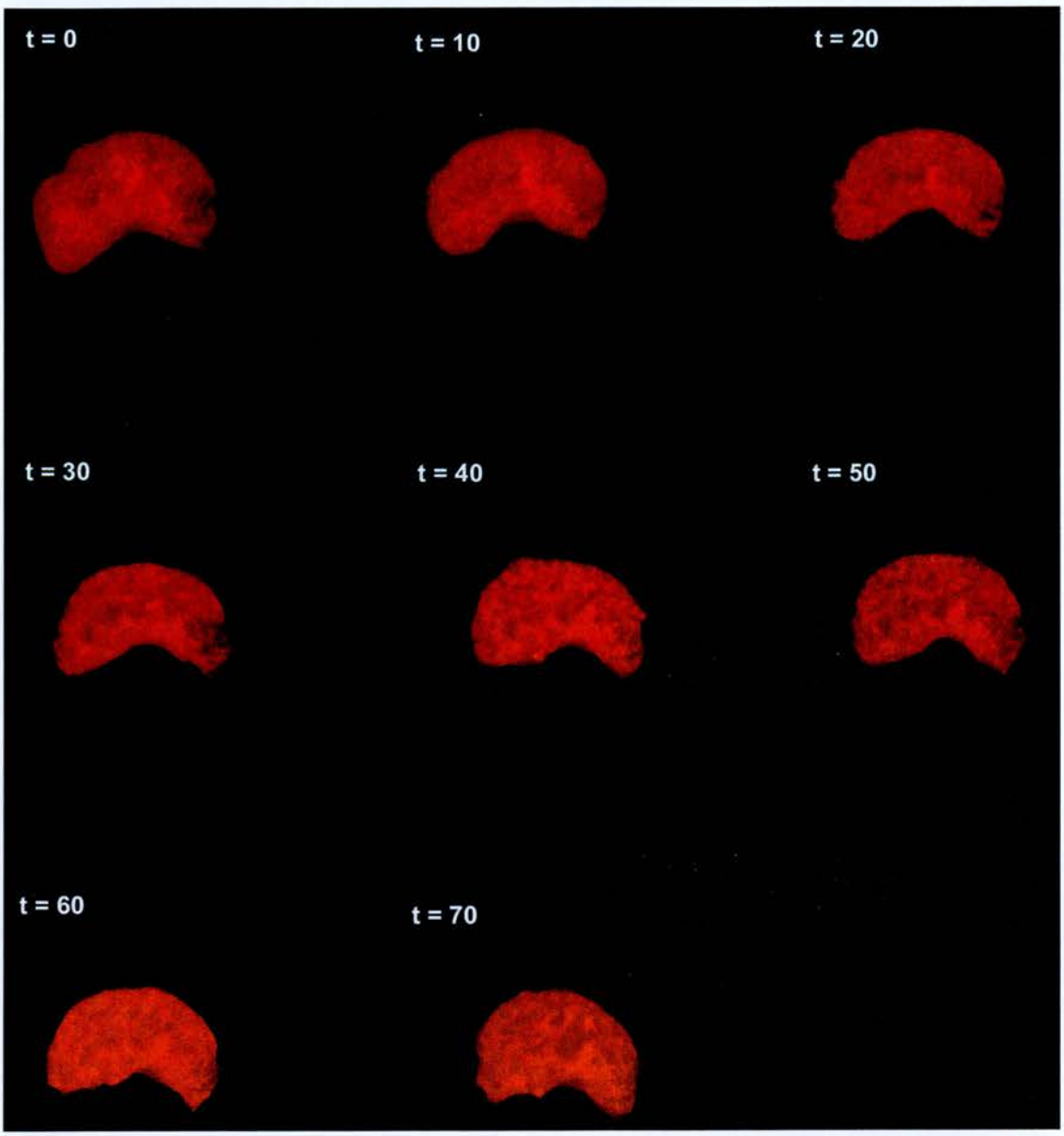


Figure 4.2a. ERα-DsRed expression in Hek 293 cells with addition of 10^{-8}M E_2. ERα-DsRed transiently transfected into Hek 293 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M E_2. Confocal images taken over 70 minutes starting from time (t) 0, at 10 minute intervals.

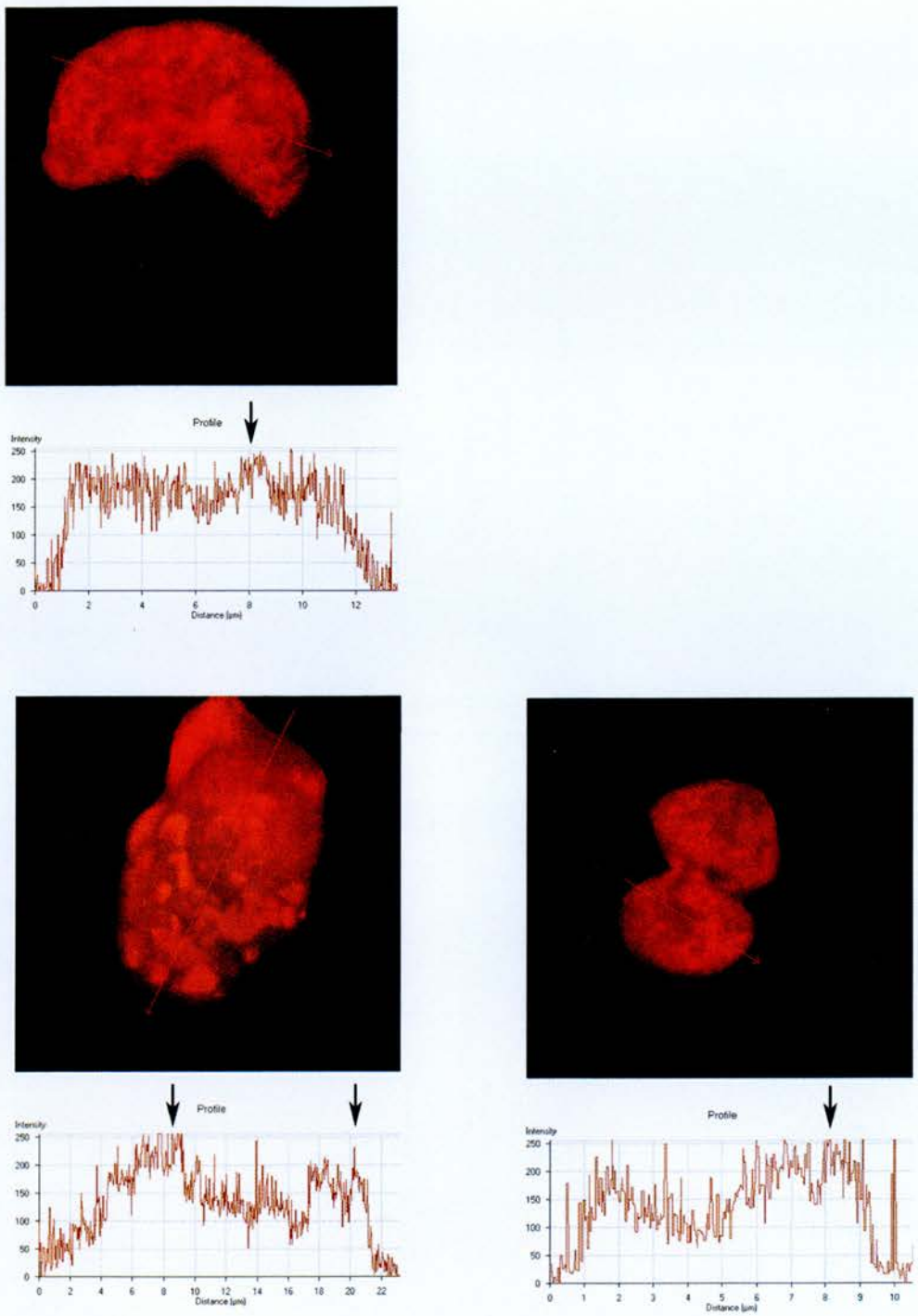


Figure 4.2b. ERα-DsRed expression in Hek 293 cells with addition of 10^{-8}M E_2 for 60 minutes. Images are of three cell nuclei showing a graph of the intensity profile of the fluorescent ER. The graph demonstrates the intensity of the fluorescence (y axis) along the length of the line drawn through the nucleus, in the direction of the arrow (x axis). All graphs shown have the same y axis values. Arrows indicate peaks in intensity.

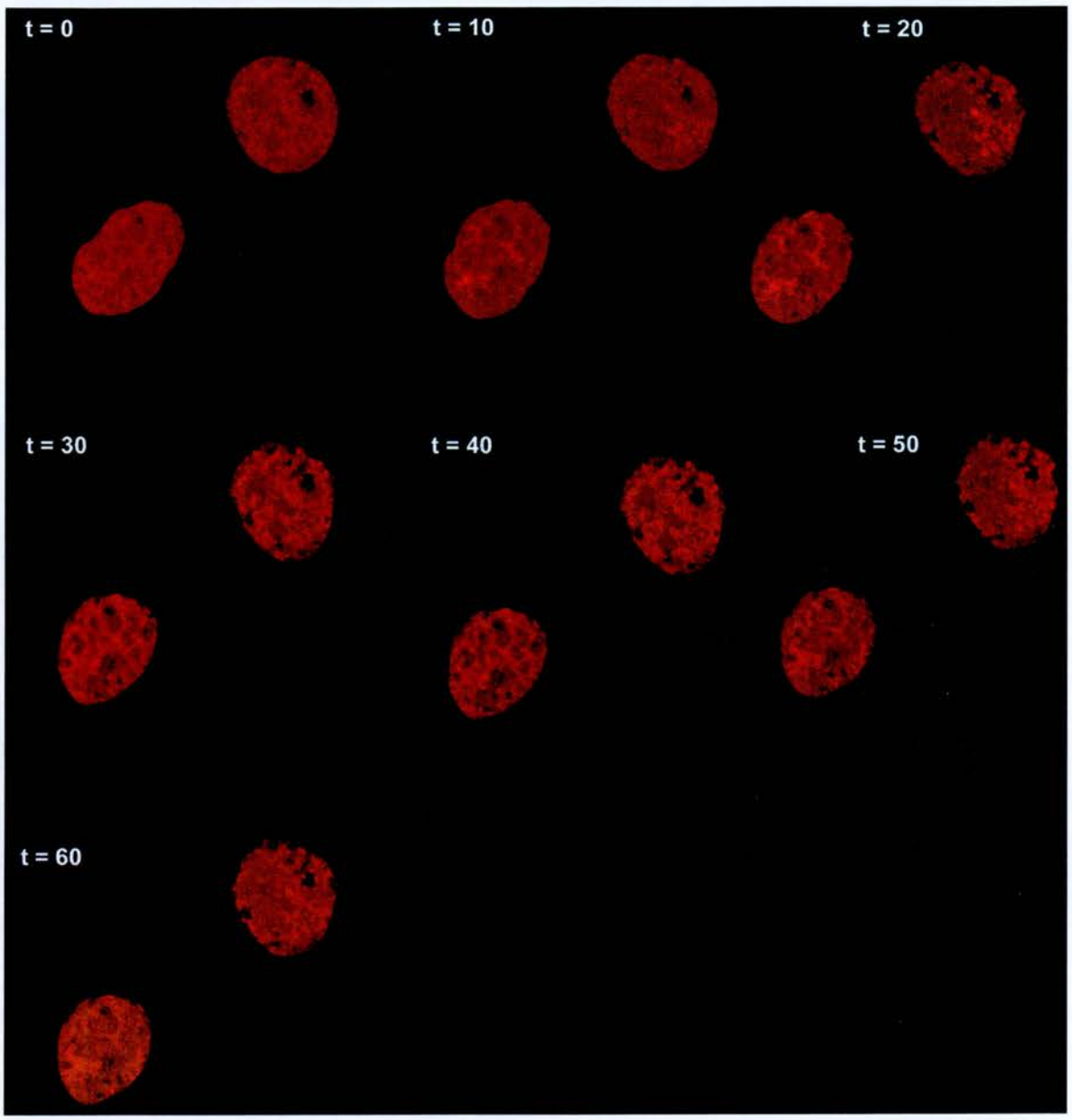


Figure 4.3a. ERα-DsRed expression in Hep G2 cells with addition of 10^{-8}M E_2. ERα-DsRed transiently transfected into Hep G2 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M E_2. Confocal images taken over 60 minutes from time (t) 0, at 10 minute intervals.

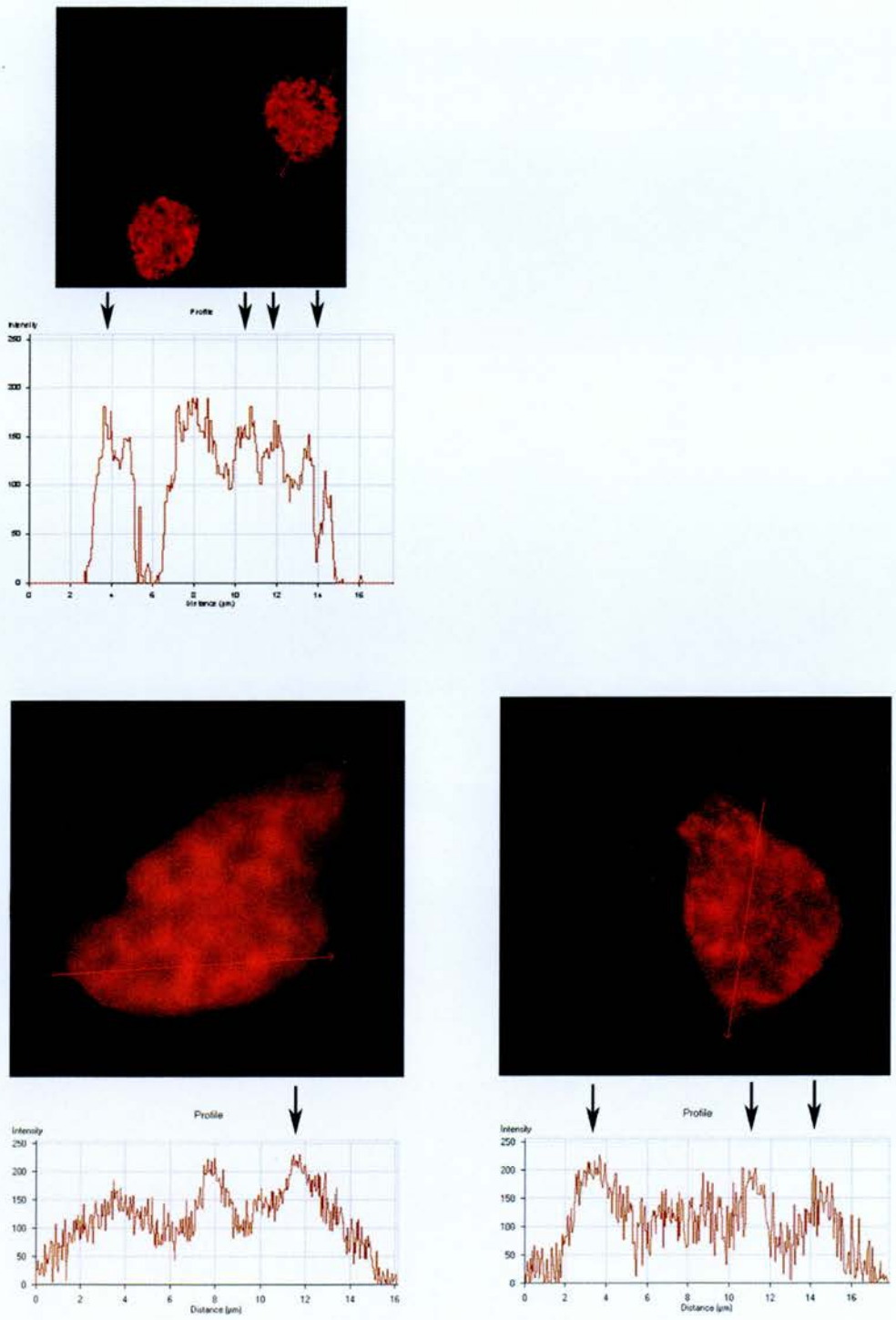


Figure 4.3b. ERα-DsRed expression in Hep G2 cells with addition of 10^{-8}M E_2 for 60 minutes. Images are of three cell nuclei showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

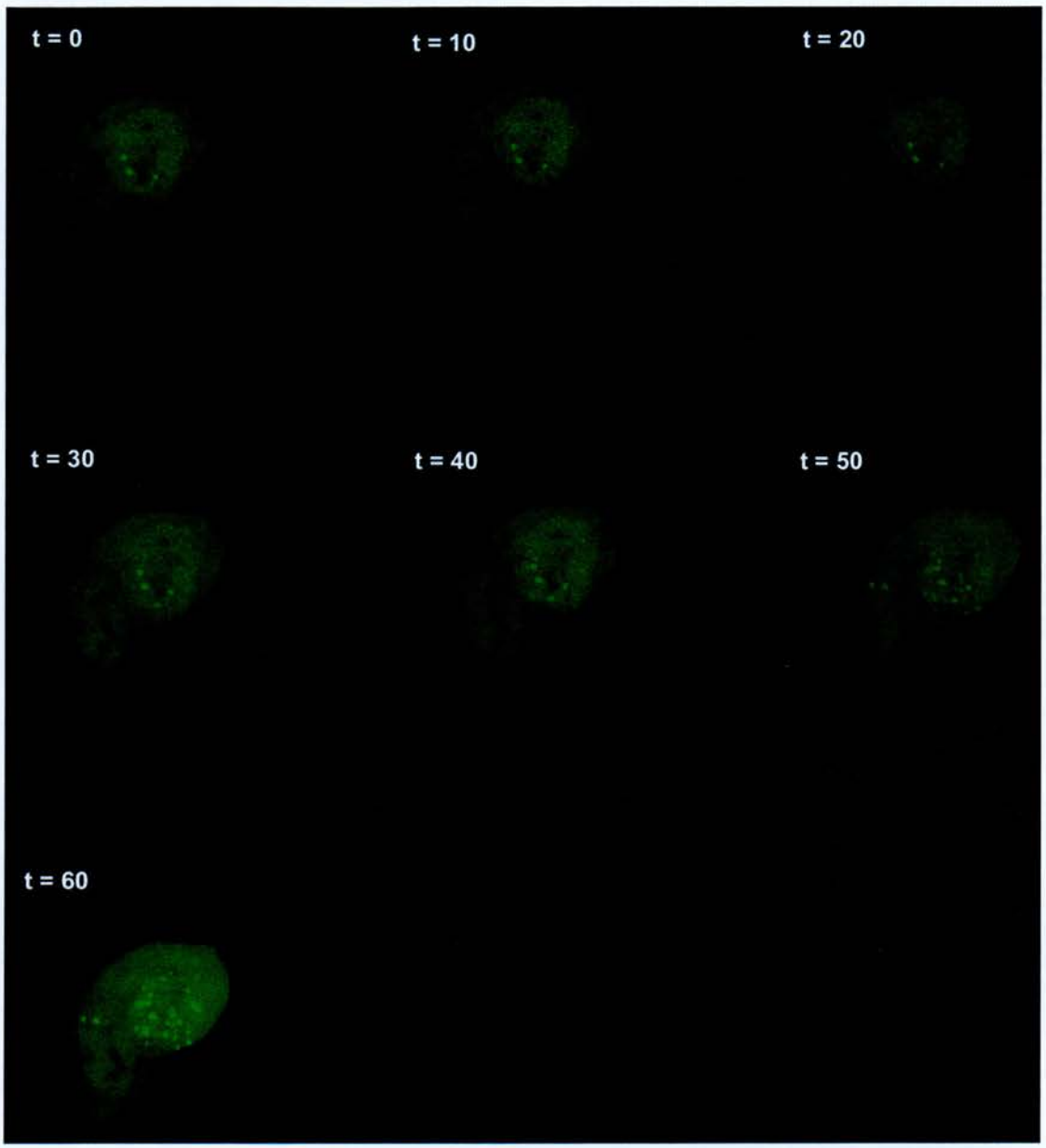


Figure 4.4a. ERβ1-EGFP expression in Hek 293 cells with addition of 10^{-8}M E_2. ERβ1-EGFP transiently transfected into Hek 293 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M E_2. Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

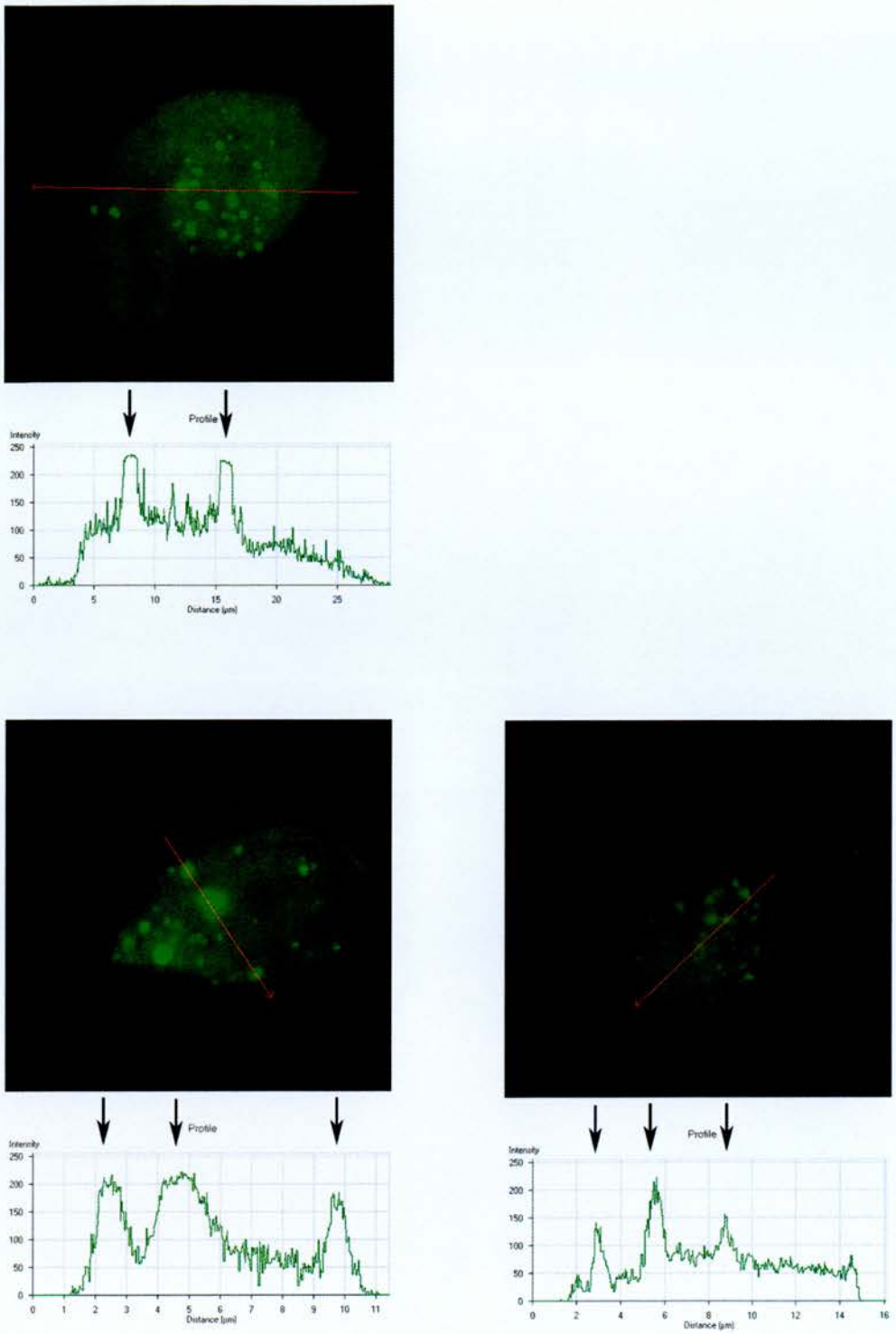


Figure 4.4b. ERβ1-EGFP expression in Hek 293 cells with addition of 10^{-8}M E_2 for 60 minutes. Images are of three cell nuclei showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

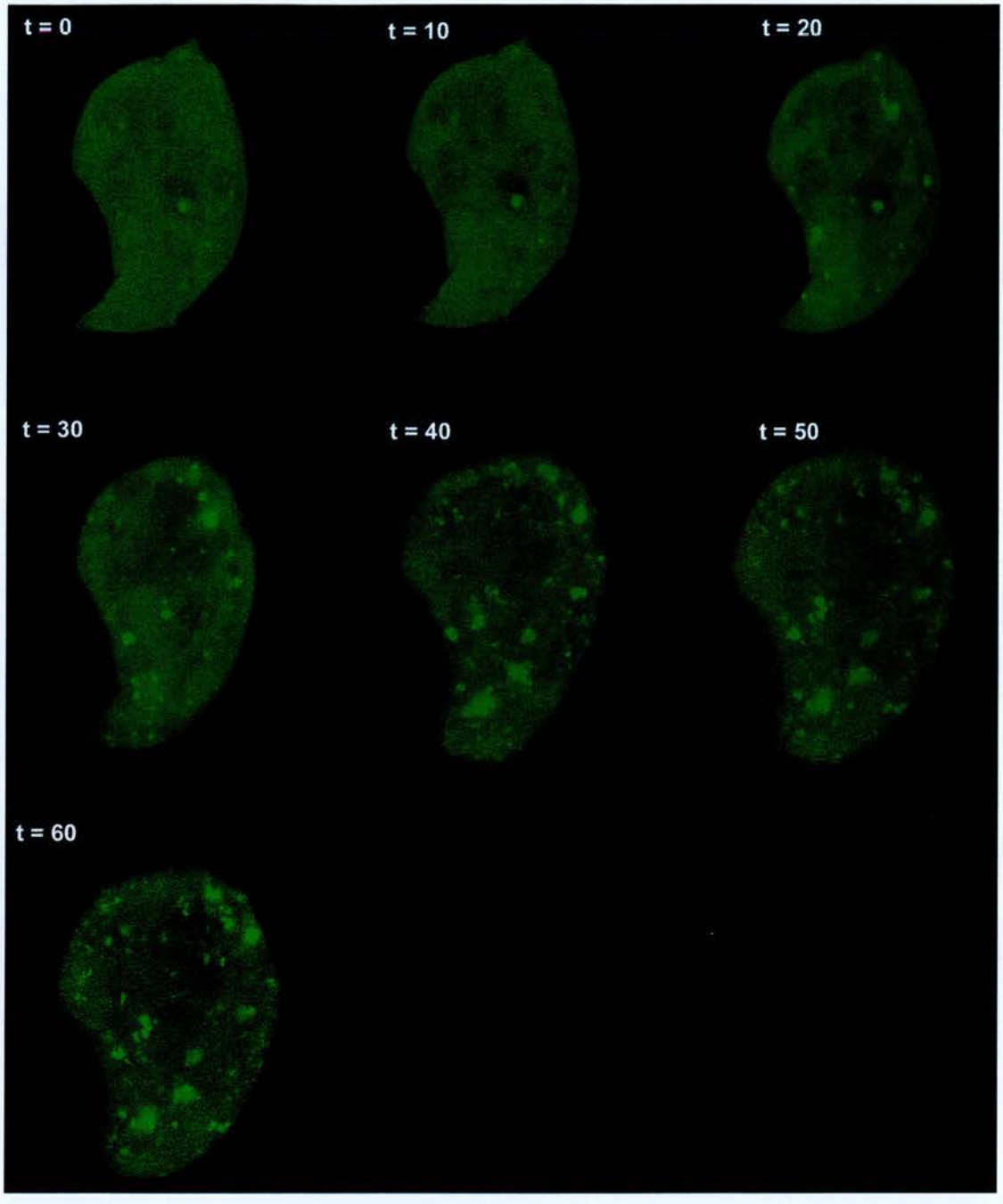


Figure 4.5a. ERβ1-EGFP expression in Hep G2 cells with addition of 10^{-8}M E_2. ERβ1-EGFP transiently transfected into Hep G2 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M E_2. Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

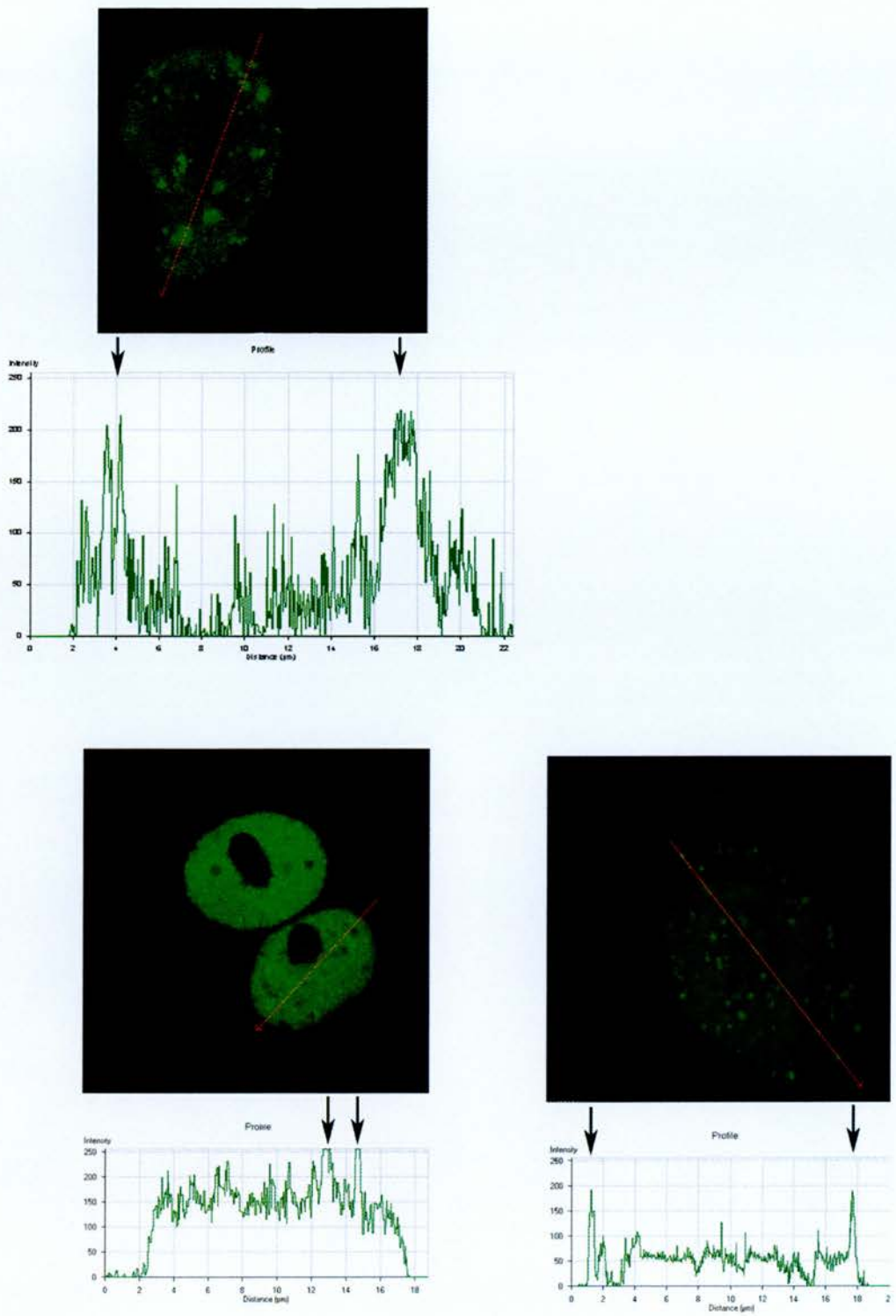


Figure 4.5b. ERβ1-EGFP expression in Hep G2 cells with addition of 10^{-8}M E_2 for 60 minutes. Images are of three cell nuclei showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

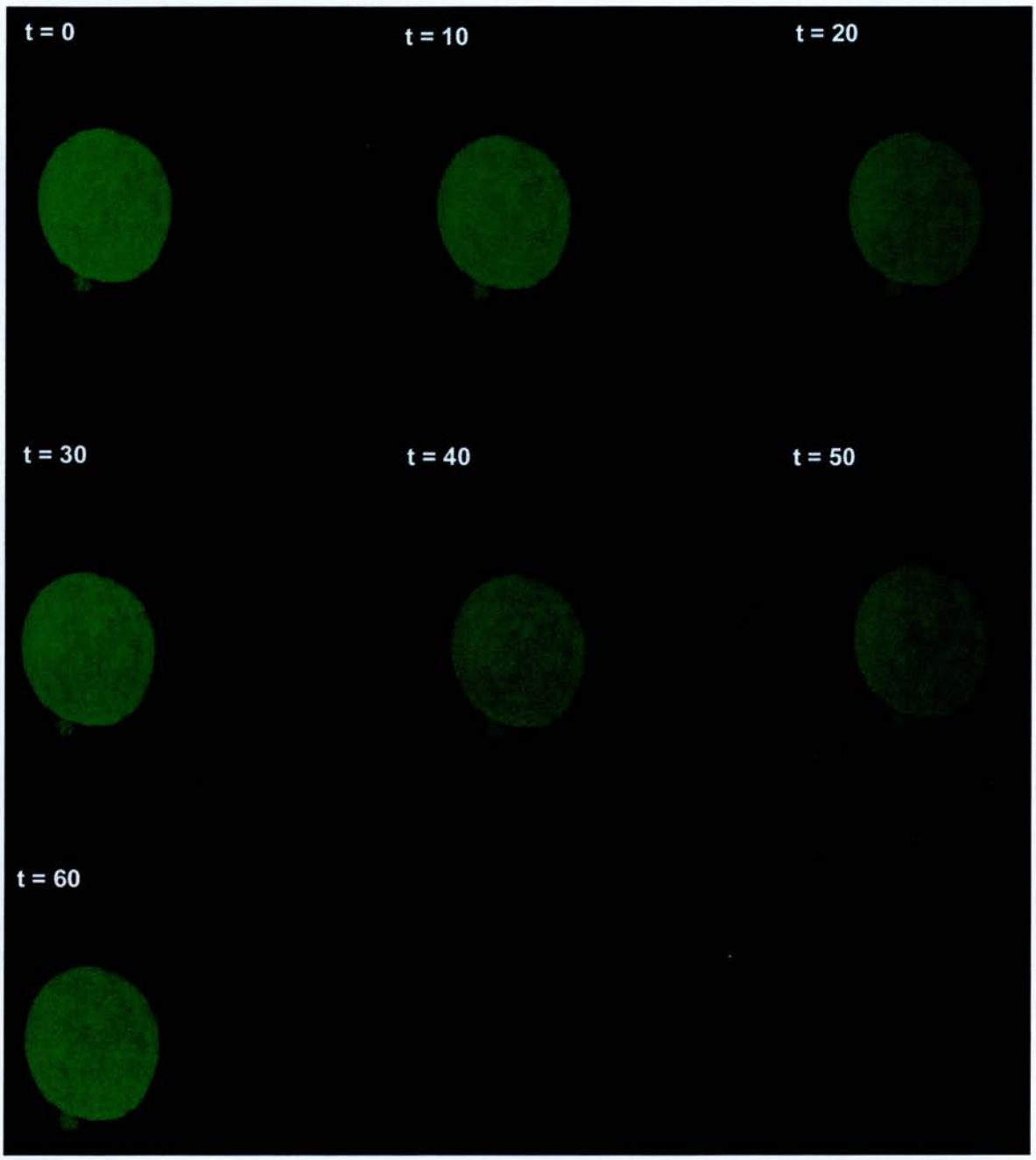


Figure 4.6a. ERβ2-EGFP expression in Hek 293 cells with addition of 10^{-8}M E_2. ERβ2-EGFP transiently transfected into Hek 293 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M E_2. Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

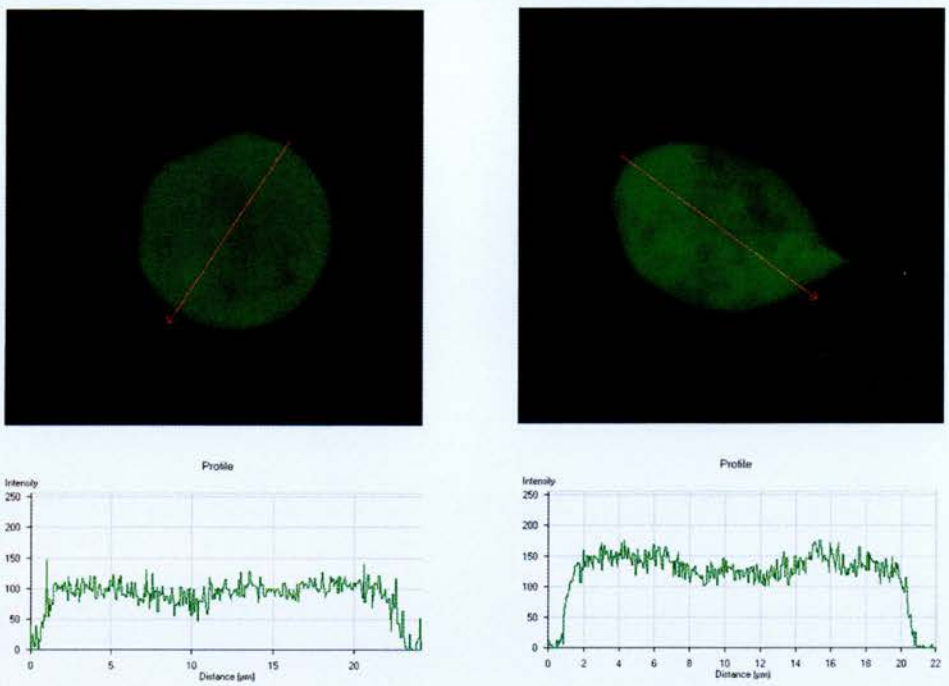


Figure 4.6b. ERβ2-EGFP expression in Hek 293 cells with addition of 10^{-8}M E_2 for 60 minutes. Images are of two cell nuclei showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

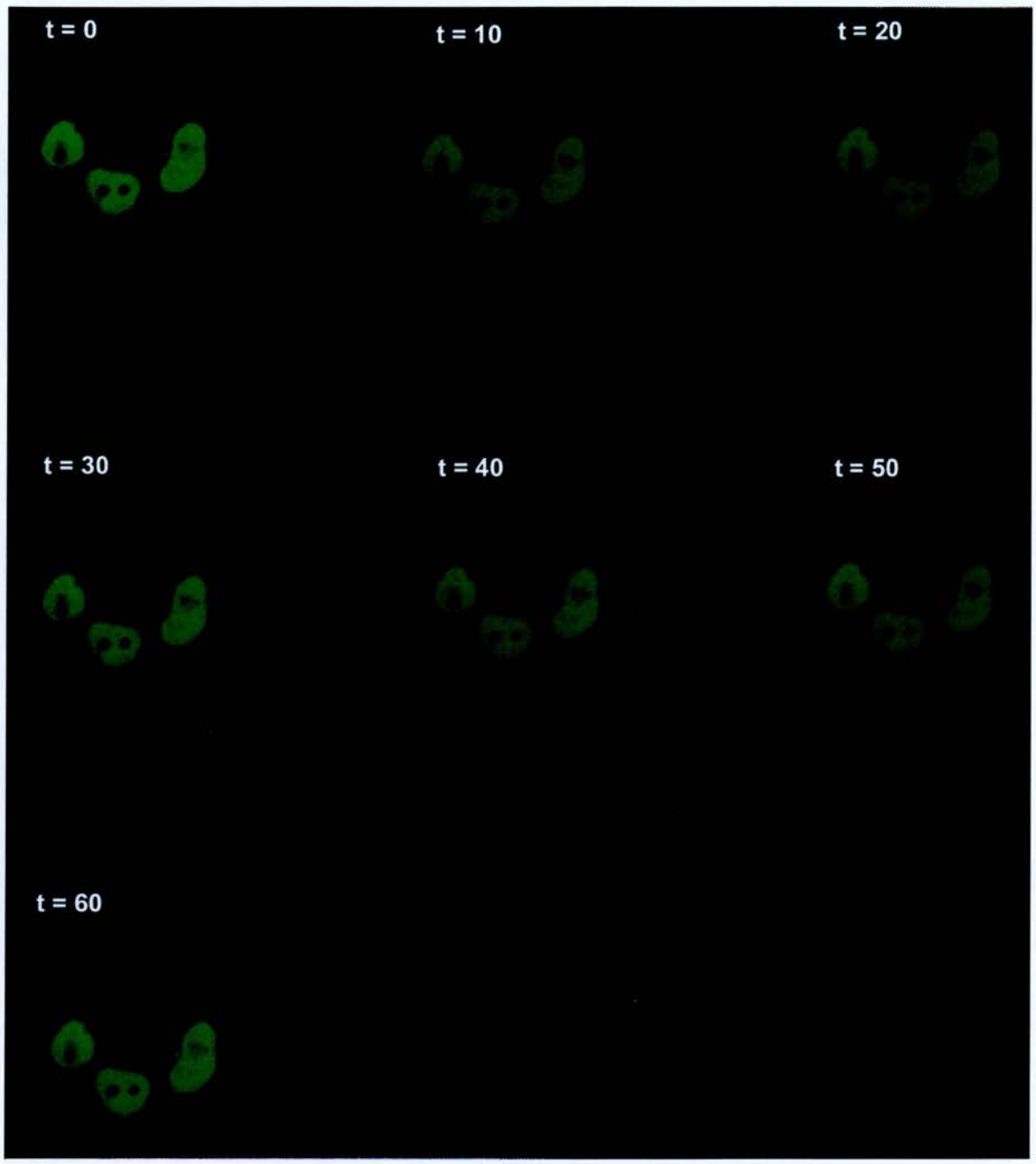


Figure 4.7a. ERβ2-EGFP expression in Hep G2 cells with addition of 10^{-8}M E_2. ERβ2-EGFP transiently transfected into Hep G2 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M E_2. Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

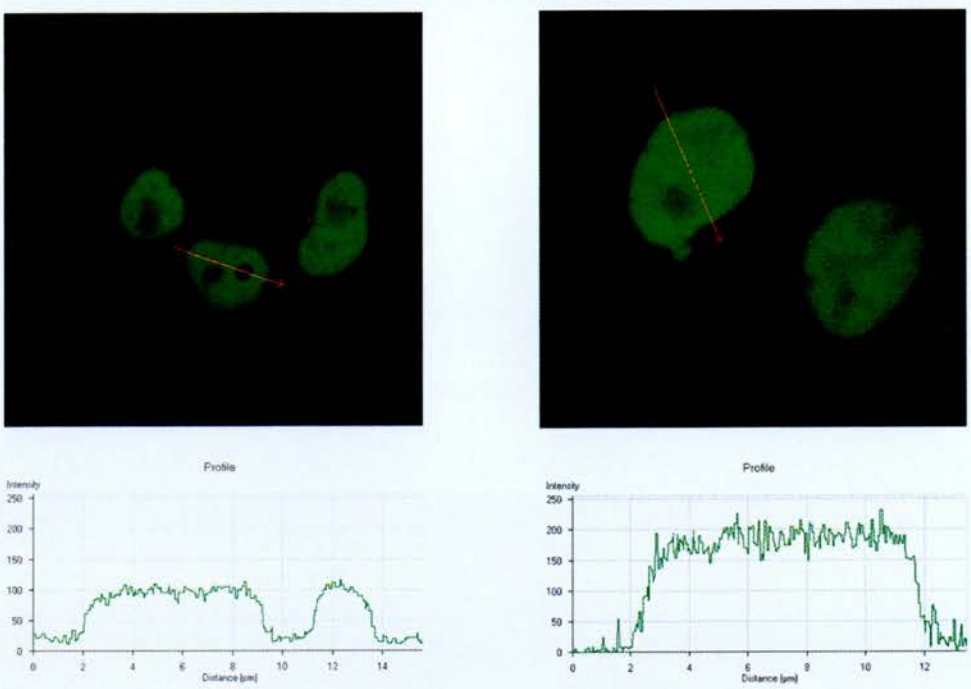


Figure 4.7b. ERβ2-EGFP expression in Hep G2 cells with addition of 10^{-8}M E_2 for 60 minutes. Images are of two cell nuclei showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

4.3.3 Treatment of Cells with 3βAdiol

In the absence of ligand ERα-DsRed adopted a diffuse distribution within the nucleus in Hek 293 and Hep G2 cells (t = 0) (*Figures 4.8a* & *4.9a*). Upon addition of 10^{-8}M 3βAdiol for 10 minutes, large foci were observed and these increased in number and brightness with time. At 30 minutes these bright foci clustered together in both cell lines, appearing as a large bright area which did not contain individual foci, as well as individual bright foci present and a decrease in the diffusely distributed ERα-DsRed (*Table 4.1* & *4.2*). The bright focal pattern was maintained in both cell lines at 60 minutes. The intensity profiles (*Figures 4.8b* & *4.9b*) show that there are differences between individual cells and the redistribution pattern indicated by the number and size of foci, with Hep G2 cells containing more discrete foci compared to Hek 293 cells.

ERβ1-EGFP was transiently transfected into Hek 293 and Hep G2 cells and treated with 10^{-8}M 3βAdiol (*Figures 4.10a* & *4.11a*). The ERβ1-EGFP remained in a diffuse pattern in the nucleus until after 40 minutes of incubation with 3βAdiol, at which time faint discrete foci appeared in Hek 293 cells. This redistribution was not observed in Hep G2 cells until 60 minutes after addition of 3βAdiol. Once ERβ1-EGFP foci appeared their brightness increased reaching a maximum at 90 minutes (Hep G2). Analysis of the intensity profiles of different cells demonstrated that at 60 minutes the number, intensity and size of the foci varied and not all of the cell nuclei that contained ERβ1-EGFP showed a redistribution of receptor following addition of 3βAdiol (*Figures 4.10b* & *4.5b*). Results are summarised in *Table 4.1 and Table 4.2*.

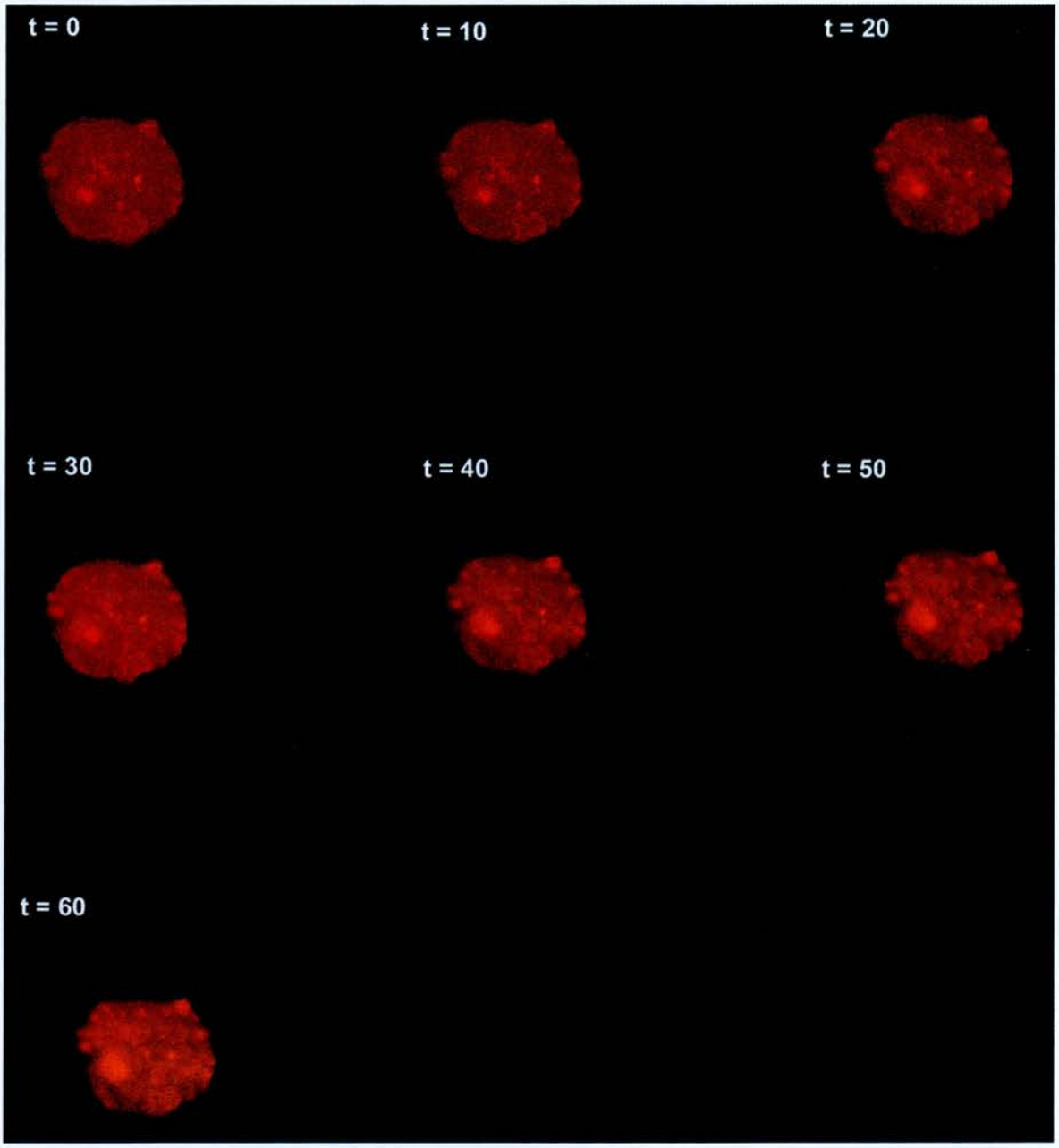


Figure 4.8a. ERα-DsRed expression in Hek 293 cells with addition of 10^{-8}M 3βAdiol. ERα-DsRed transiently transfected into Hek 293 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M 3βAdiol. Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

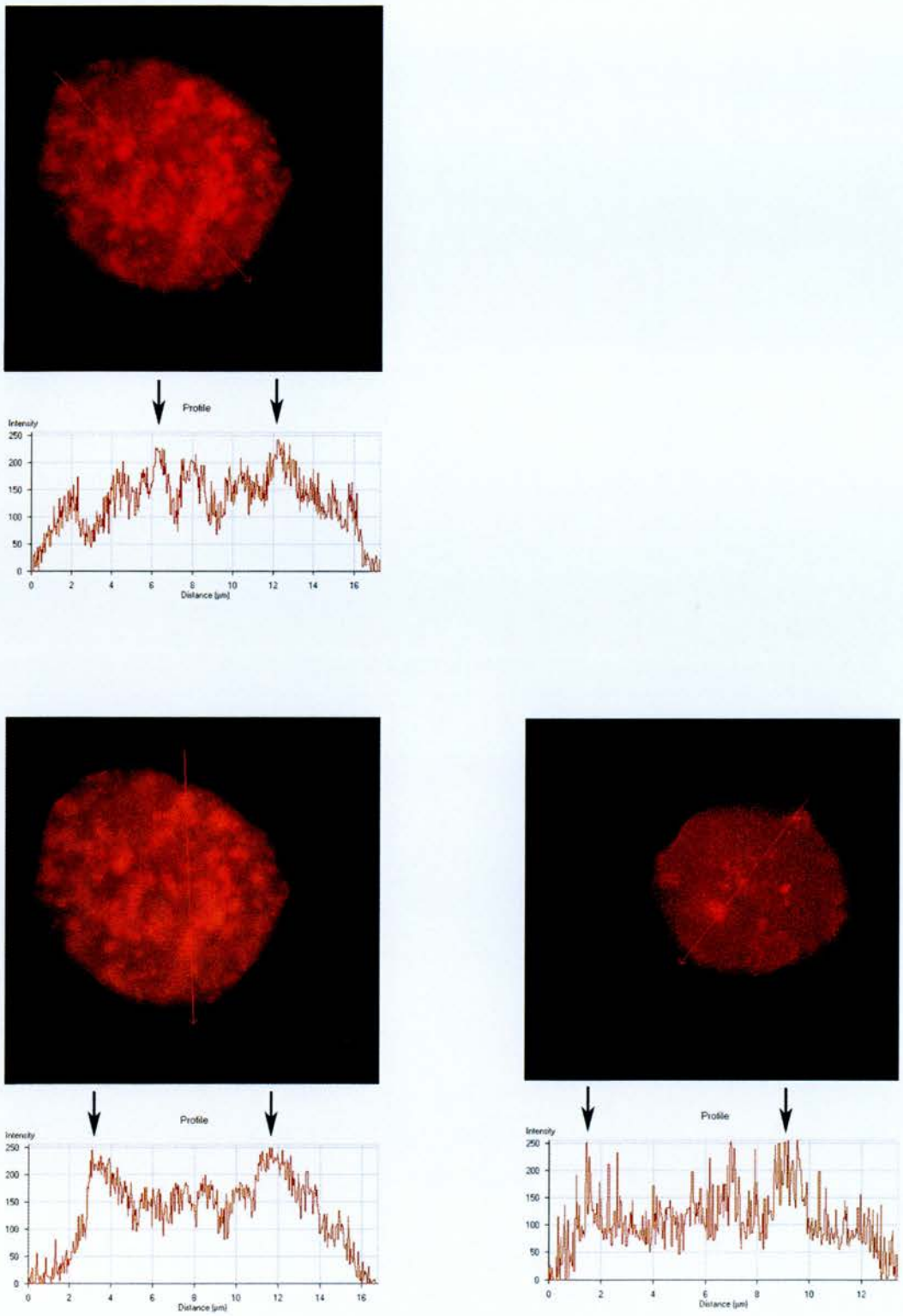


Figure 4.8b. ERα-DsRed expression in Hek 293 cells with addition of 10^{-8}M 3βAdiol for 60 minutes. Images are of three cell nuclei showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

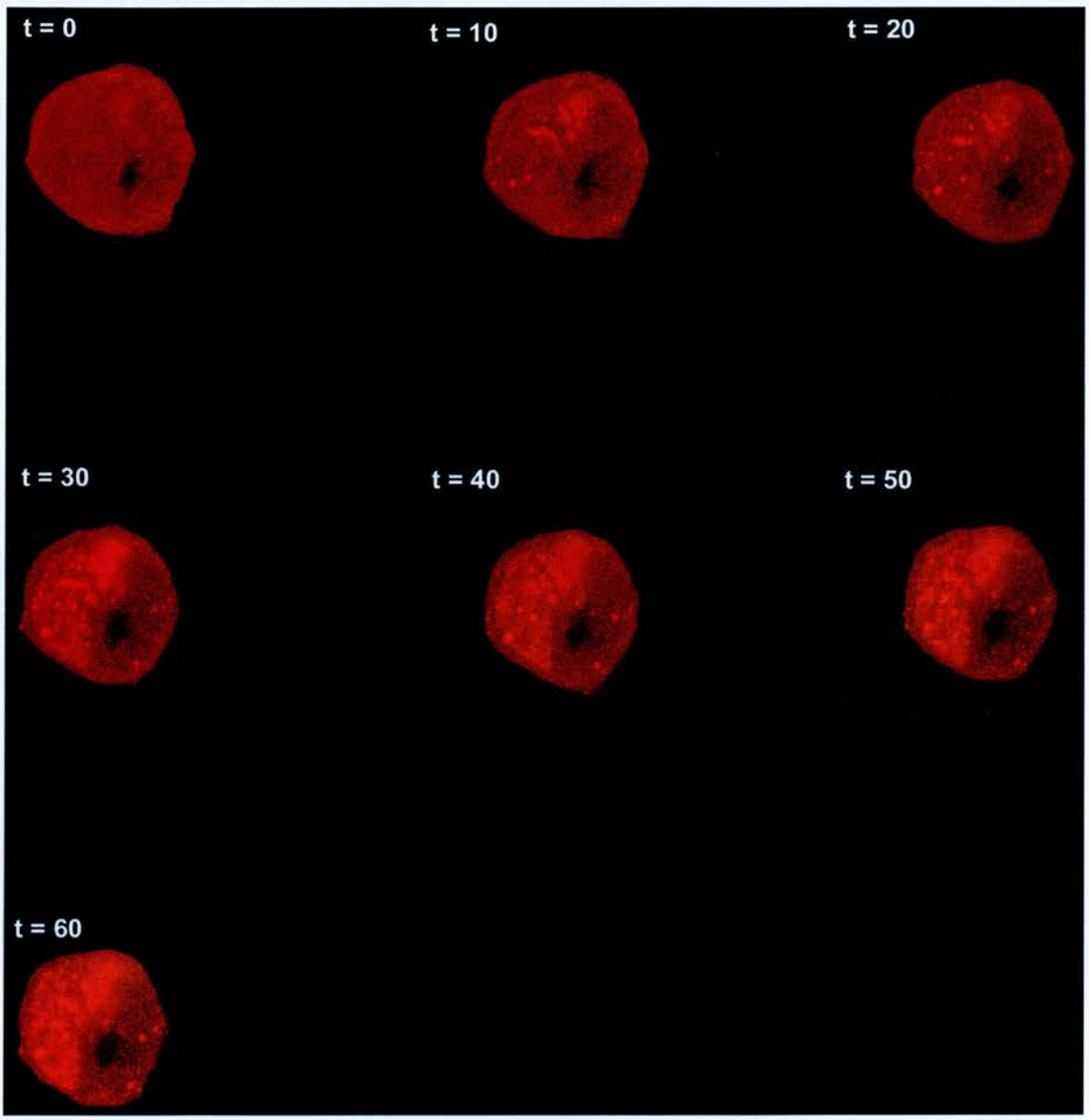


Figure 4.9a. ERα-DsRed expression in Hep G2 cells with addition of 10^{-8}M 3βAdiol. ERα-DsRed transiently transfected into Hep G2 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M 3βAdiol. Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

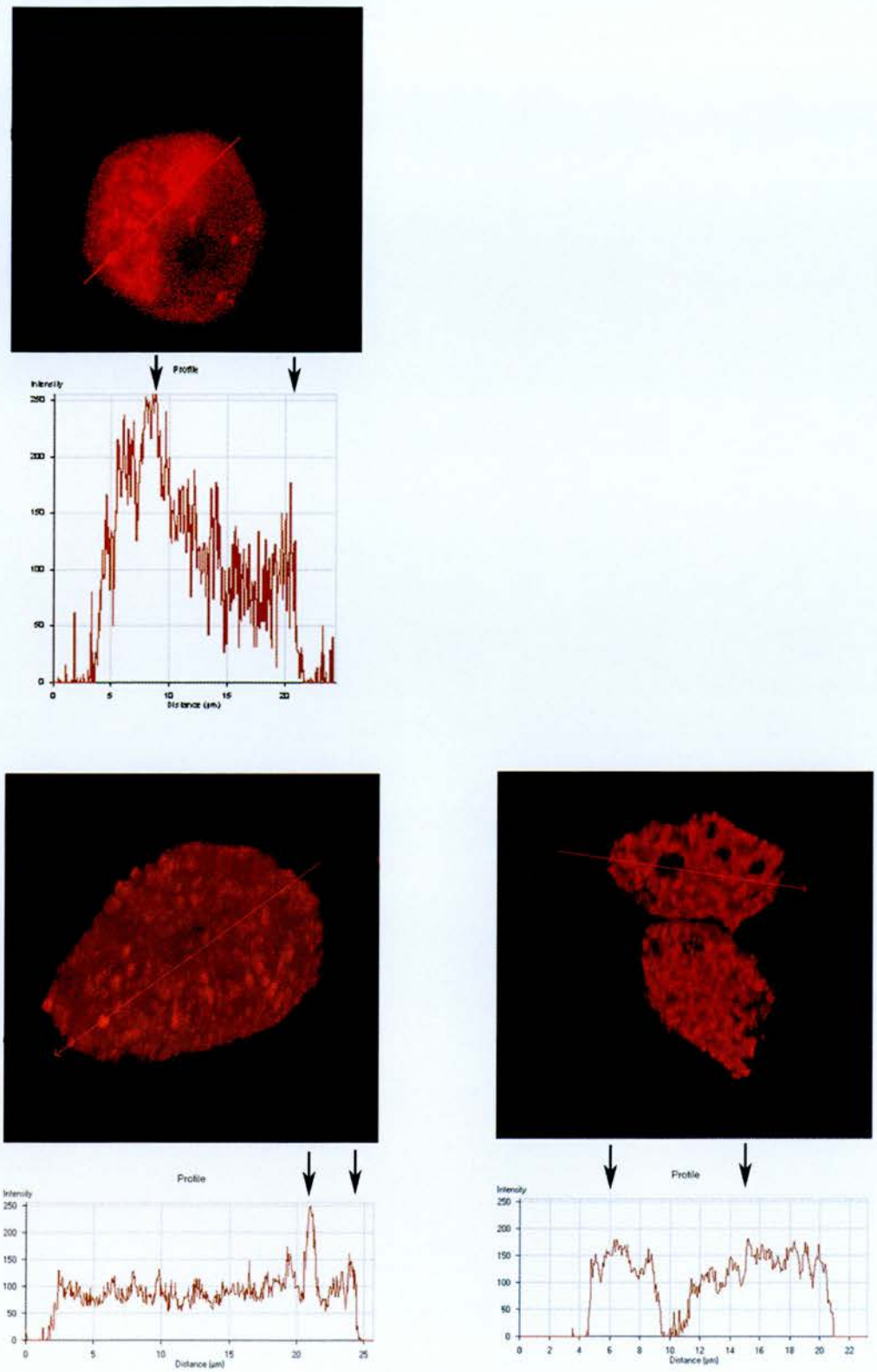


Figure 4.9b. ERα-DsRed expression in Hep G2 cells with addition of 10^{-8}M 3βAdiol for 60 minutes. Images are of three cell nuclei showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

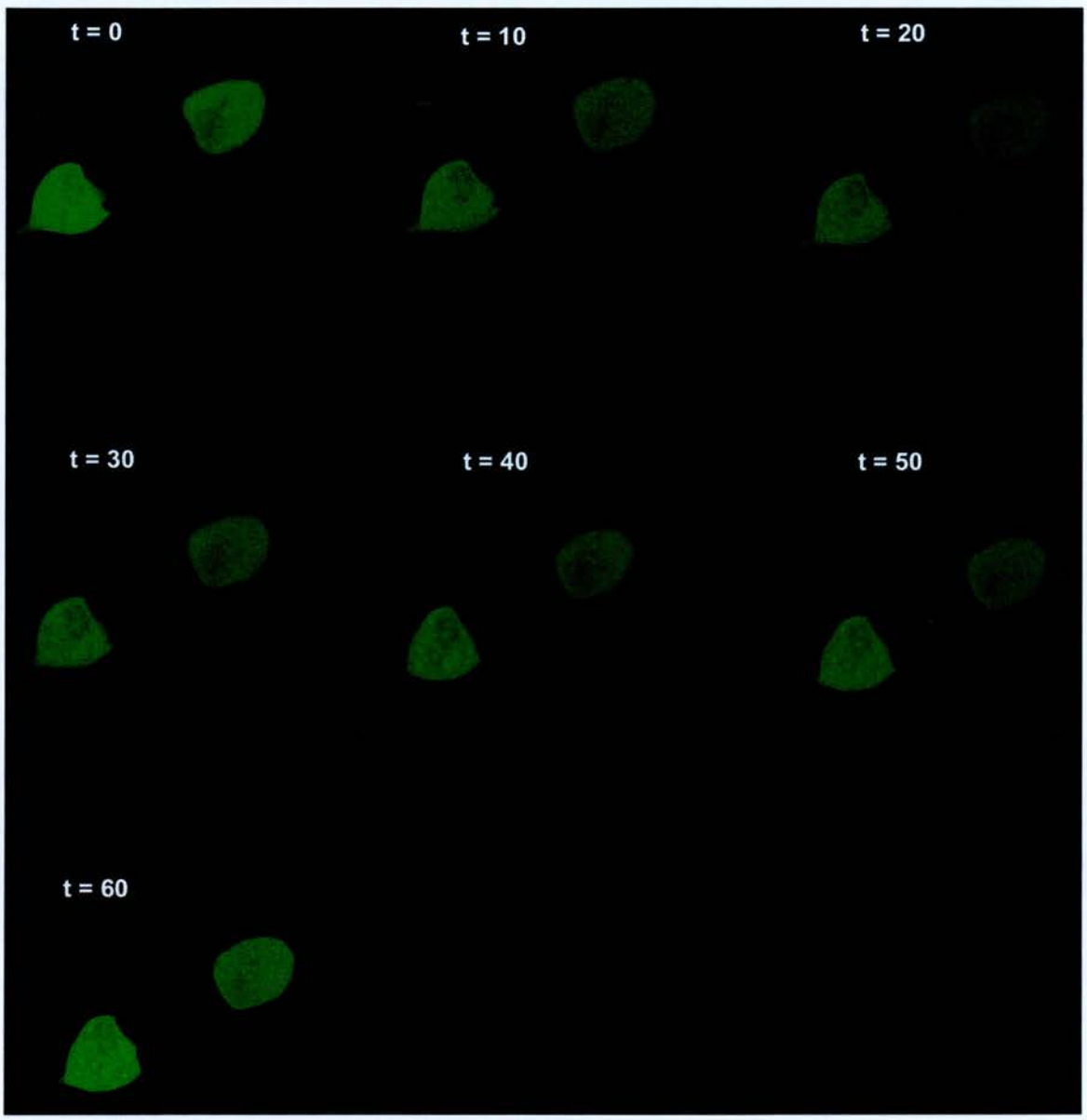


Figure 4.10a. ERβ1-EGFP expression in Hek 293 cells with addition of 10^{-8}M 3βAdiol. ERβ1-EGFP transiently transfected into Hek 293 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M 3βAdiol. Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

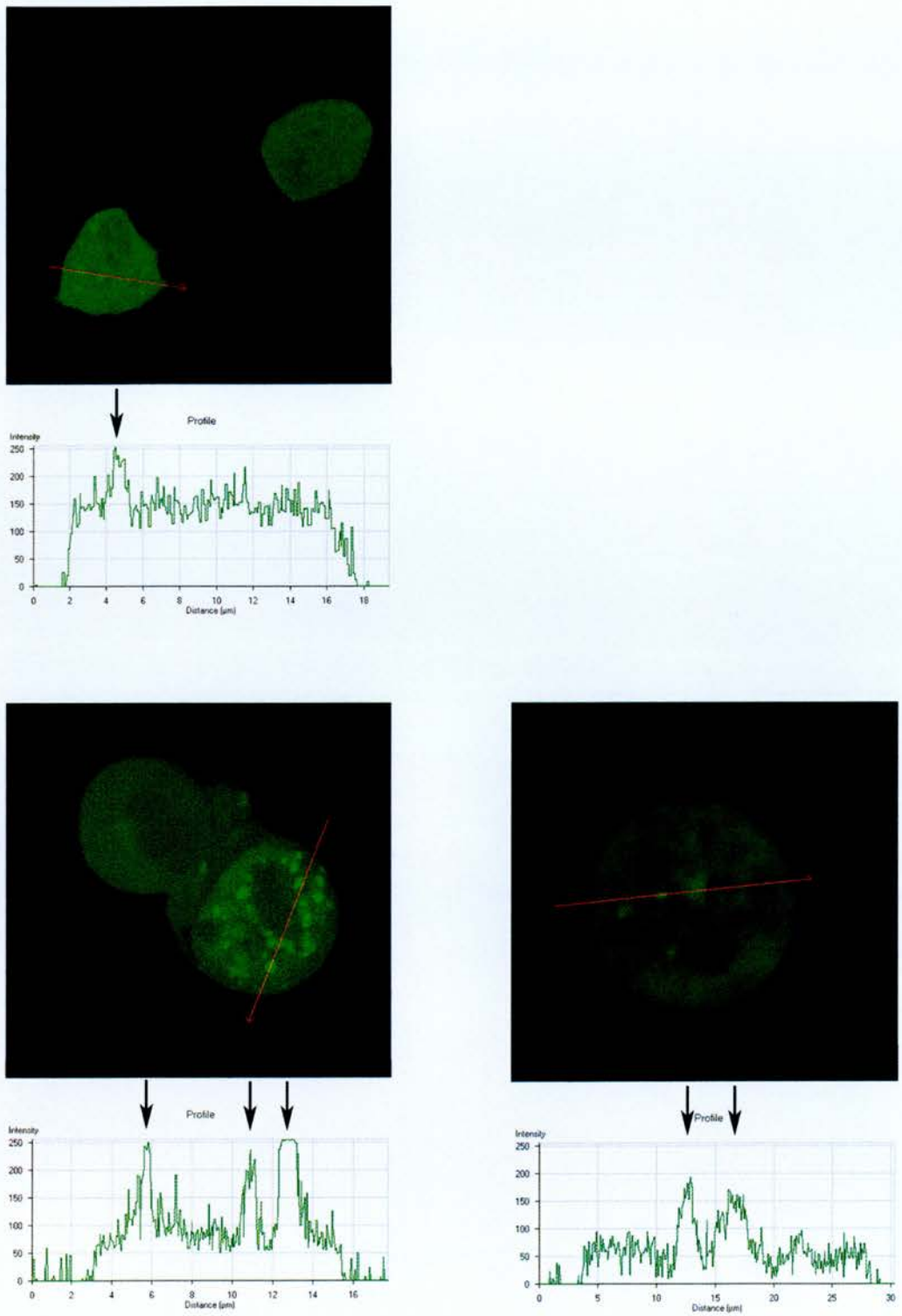


Figure 4.10b. ERβ1-EGFP expression in Hek 293 cells with addition of 10^{-8}M 3βAdiol for 60 minutes. Images are of three cell nuclei showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

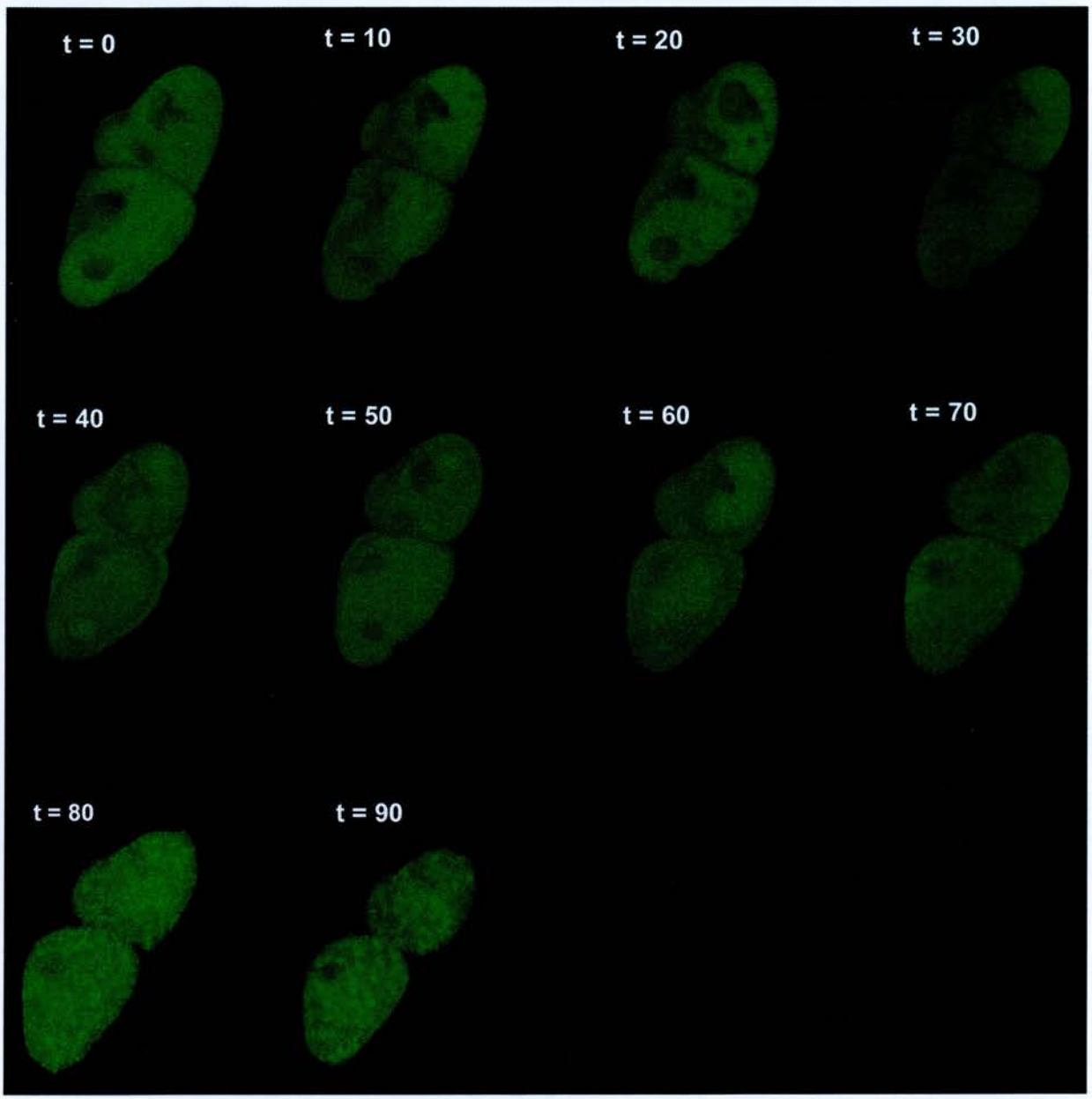


Figure 4.11a. ERβ1-EGFP expression in Hep G2 cells with addition of 10^{-8}M 3βAdiol. ERβ1-EGFP transiently transfected into Hep G2 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M 3βAdiol. Confocal images taken over 90 minutes starting from time (t) 0, at 10 minute intervals.

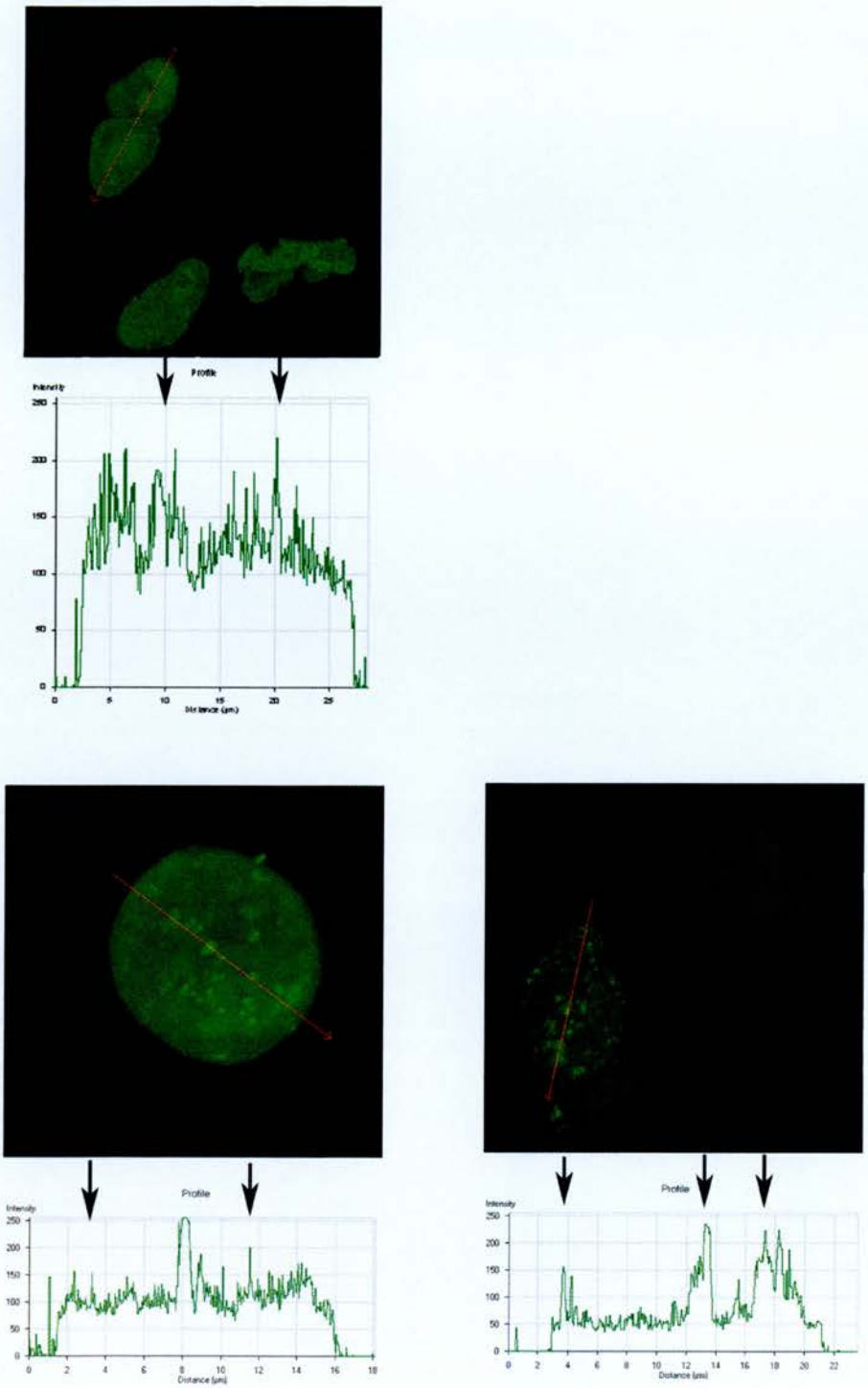


Figure 4.11b. ERβ1-EGFP expression in Hep G2 cells with addition of 10^{-8}M 3βAdiol for 60 minutes. Images are of three cell nuclei showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

4.3.4 Treatment of Cells with Genistein

ERα-DsRed transiently transfected into Hep G2 cells (*Figure 4.12a*) showed redistribution upon addition of 10^{-8}M genistein. Prior to the addition of ligand, ERα-DsRed was in a diffuse pattern with slightly brighter/darker areas within the nucleus. After 10 minutes with genistein, the receptor redistributed and brighter areas appeared, however it was not possible to observe individual foci. After 40 minutes there were definite bright areas and a reduction in the diffuse ERα-DsRed. The focal areas were most defined between 40 to 90 minutes after addition of genistein (see *Table 4.2*). After 110 minutes the foci migrated to the edge of the nucleus and the nucleus appeared to reduce in size, possibly due to nutrient depletion. The intensity profile (*Figure 4.12b*) indicates that the time lapse redistribution pattern at 60 minutes is typical for other cells and that individual foci are difficult to pick out.

ERβ1-EGFP transiently transfected into Hep G2 cells (*Figure 4.13a*) redistributed upon the addition of 10^{-8}M genistein. With no ligand present, ERβ1-EGFP was uniformly distributed within the Hep G2 cell nucleus. After 20 minutes the ERβ1-EGFP redistributed into darker areas and faint discrete foci appeared. After 30 minutes the foci increased in number and intensity, reaching a maximum at 60 minutes. The intensity profiles (*Figure 4.13b*) demonstrate variations between cells and their number of visible foci, ERα-DsRed redistributed into large bright foci whereas ERβ1-EGFP redistributed into more discrete foci. Results are summarised in *Table 4.2*.

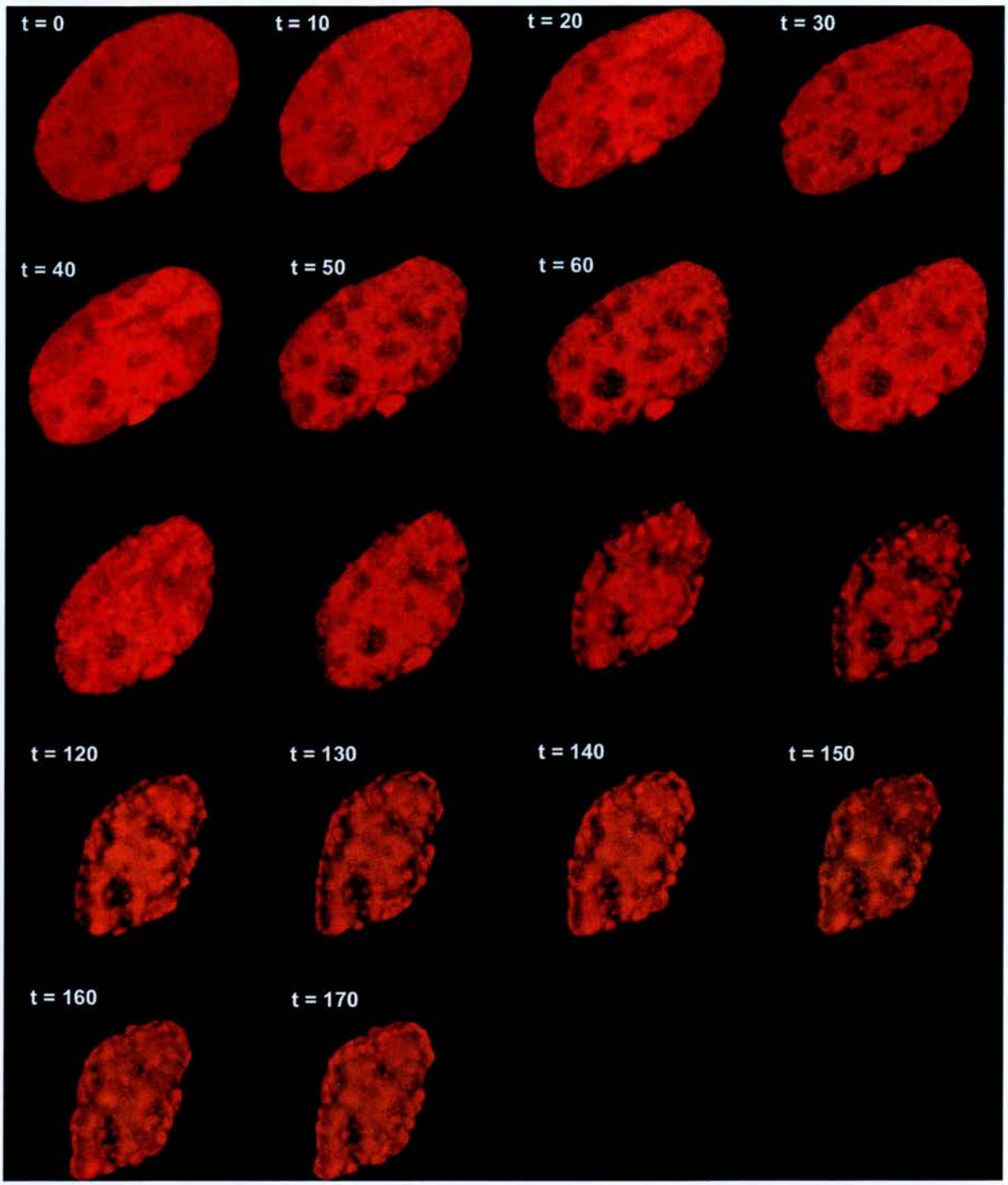


Figure 4.12a. ERα-DsRed expression in Hep G2 cells with addition of 10^{-8}M genistein. ERα-DsRed transiently transfected into Hep G2 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M genistein. Confocal images taken over 170 minutes starting from time (t) 0, at 10 minute intervals.

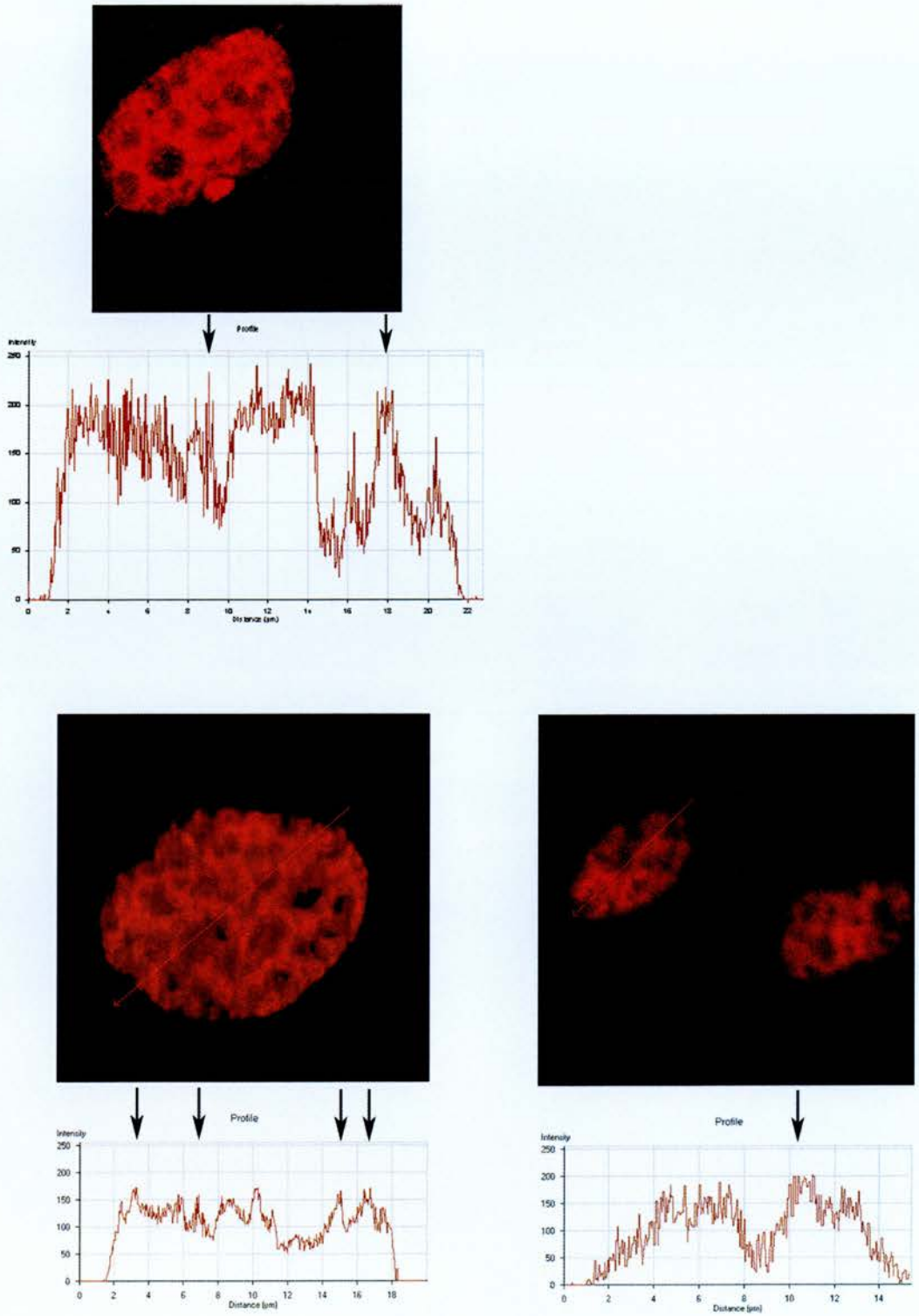


Figure 4.12b. ERα-DsRed expression in Hep G2 cells with addition of 10^{-8}M genistein for 60 minutes. Images are of three cell nuclei showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

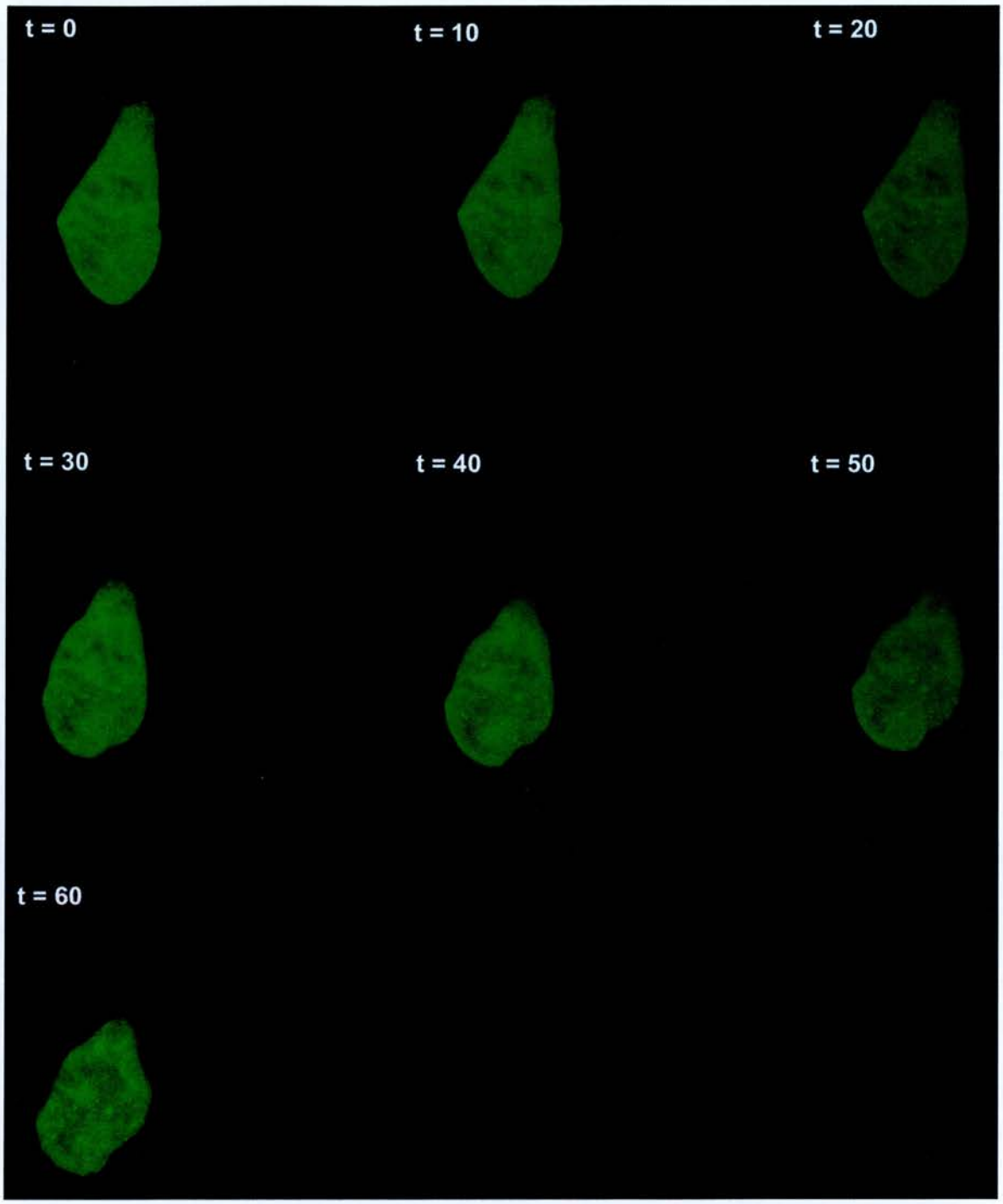


Figure 4.13a. ERβ1-EGFP expression in Hep G2 cells with addition of 10^{-8}M genistein. ERβ1-EGFP transiently transfected into Hep G2 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M genistein. Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

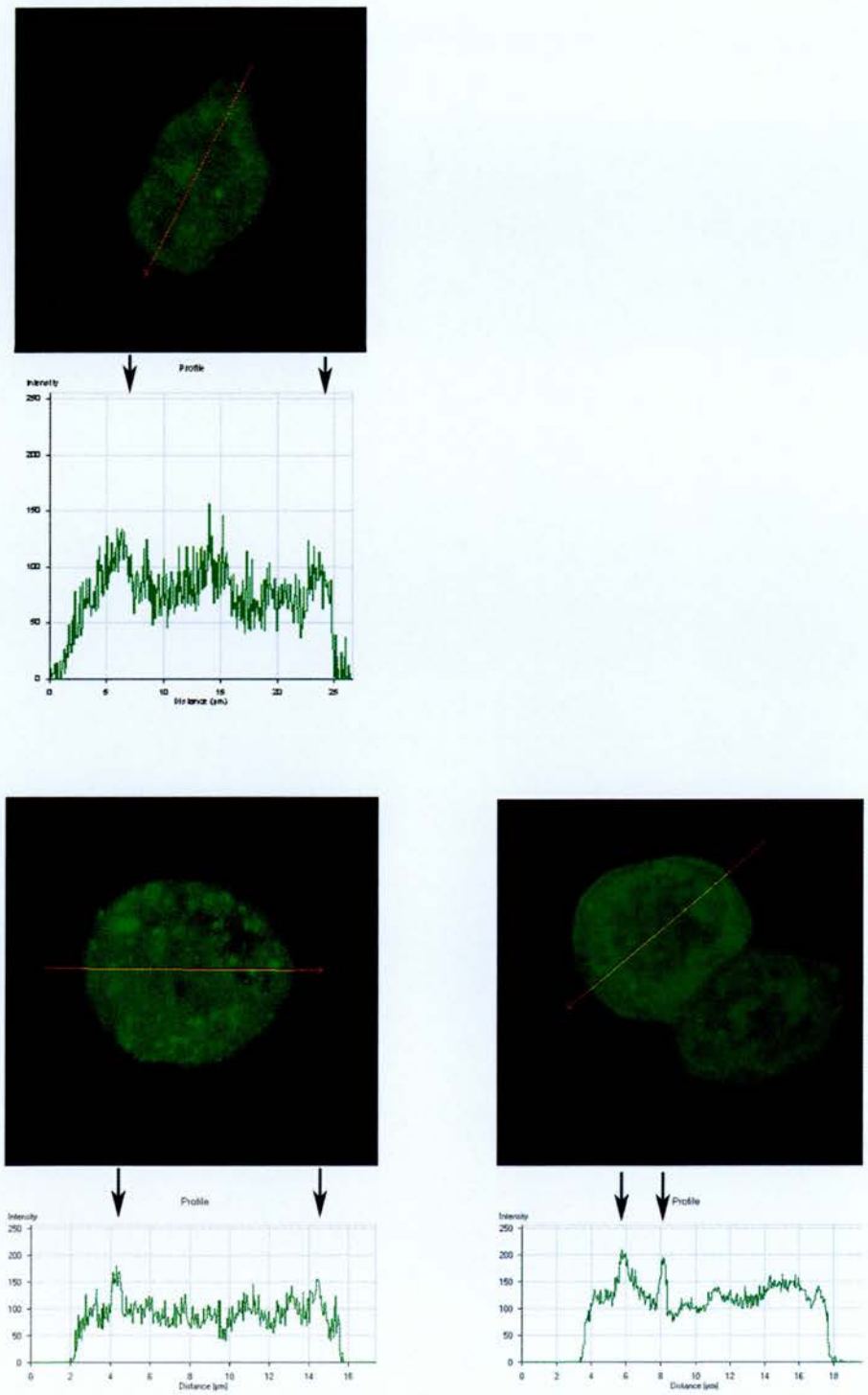


Figure 4.13b. ERβ1-EGFP expression in Hep G2 cells with addition of 10^{-8}M genistein for 60 minutes. Images are of three cell nuclei showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

4.3.5 Treatment of Cells with PPT™

The commercial ERα agonist PPT™ activated transiently transfected ERα-DsRed in Hep G2 cells (*Figure 4.14a*). In the absence of ligand the ERα-DsRed was diffusely distributed with a few brighter areas. Upon addition of 10^{-8}M PPT™ these brighter areas increased in intensity and at 30 minutes large but discrete foci were observed, with a decrease in the diffuse ERα-DsRed present in the nucleus. After 50 minutes the maximum redistribution of ERα-DsRed was observed with the least amount of diffuse ERα and maximal numbers of bright foci. The intensity profiles (*Figure 4.14b*) demonstrates the redistribution of the ERα into either bright areas or more discrete foci. Results are summarised in *Table 4.2*.

ERβ1-EGFP transiently transfected in Hep G2 cells (*Figure 4.15a*) did not redistribute from a diffuse pattern on addition of 10^{-8}M PPT™ after 60 minutes.

4.3.6 Treatment of Cells with DPN™

Addition of the commercial ERβ1 agonist DPN™ altered the distribution of ERα-DsRed transiently transfected into Hep G2 cells (*Figure 4.16a*). Prior to 10^{-8}M DPN™ being added to the cells, ERα-DsRed had a mainly diffuse pattern of expression within the nucleus, with the exception of a few brighter areas present. Once 10^{-8}M DPN™ had been added to the cells more foci appeared, increasing in number and intensity to reach a maximal difference between foci and diffuse ERα-DsRed after 40 minutes (*Table 4.2*). At this time point the uniform ERα-DsRed distribution was not observed, instead ERα-DsRed appeared as bright and dark areas where very little diffuse ERα-DsRed was present. After 100 minutes there were still very bright large foci present, but these started to re-locate to a more uniform distribution. The redistribution of ERα upon addition of DPN™ varied between cells as shown in the intensity profiles (*Figure 4.16b*), the individual foci were either visible or appeared as a larger bright area.

ERβ1-EGFP in Hep G2 cells (*Figure 4.17a*) redistributed upon addition of 10^{-8}M DPN™, however the formation of discrete foci did not occur until after 50 minutes of incubation. The number of foci increased in number and brightness with time, reaching a maximum at 70 minutes, after which they remained the same. The intensity profiles (*Figure 4.17b*) showed the ERβ1-EGFP redistributed to different extents in different cells and very discrete foci were observable unlike with ERα.

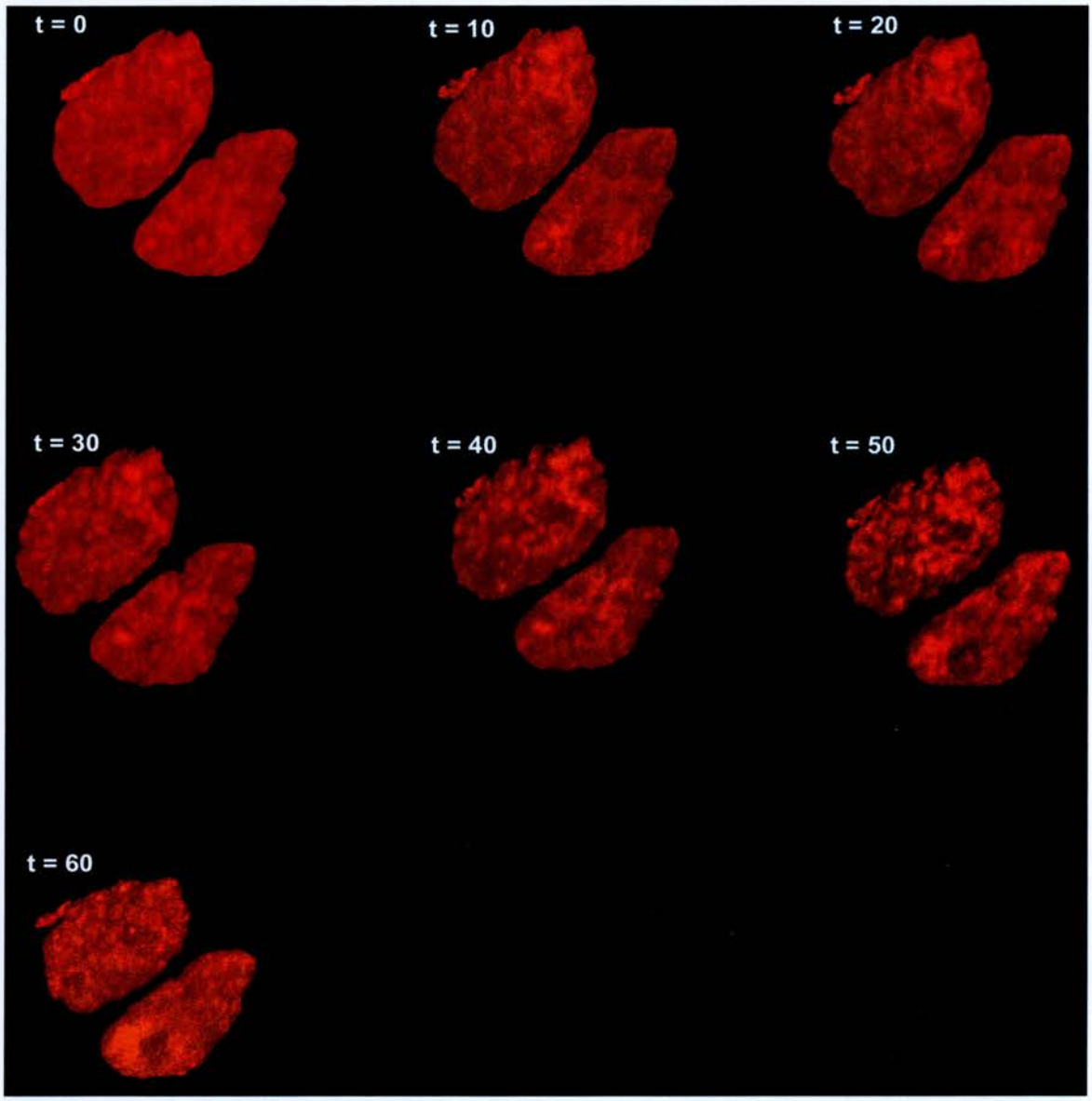


Figure 4.14a. ERα-DsRed expression in Hep G2 cells with addition of 10^{-8}M PPT™. The ERα-DsRed construct transiently transfected into Hep G2 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M PPT™. Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

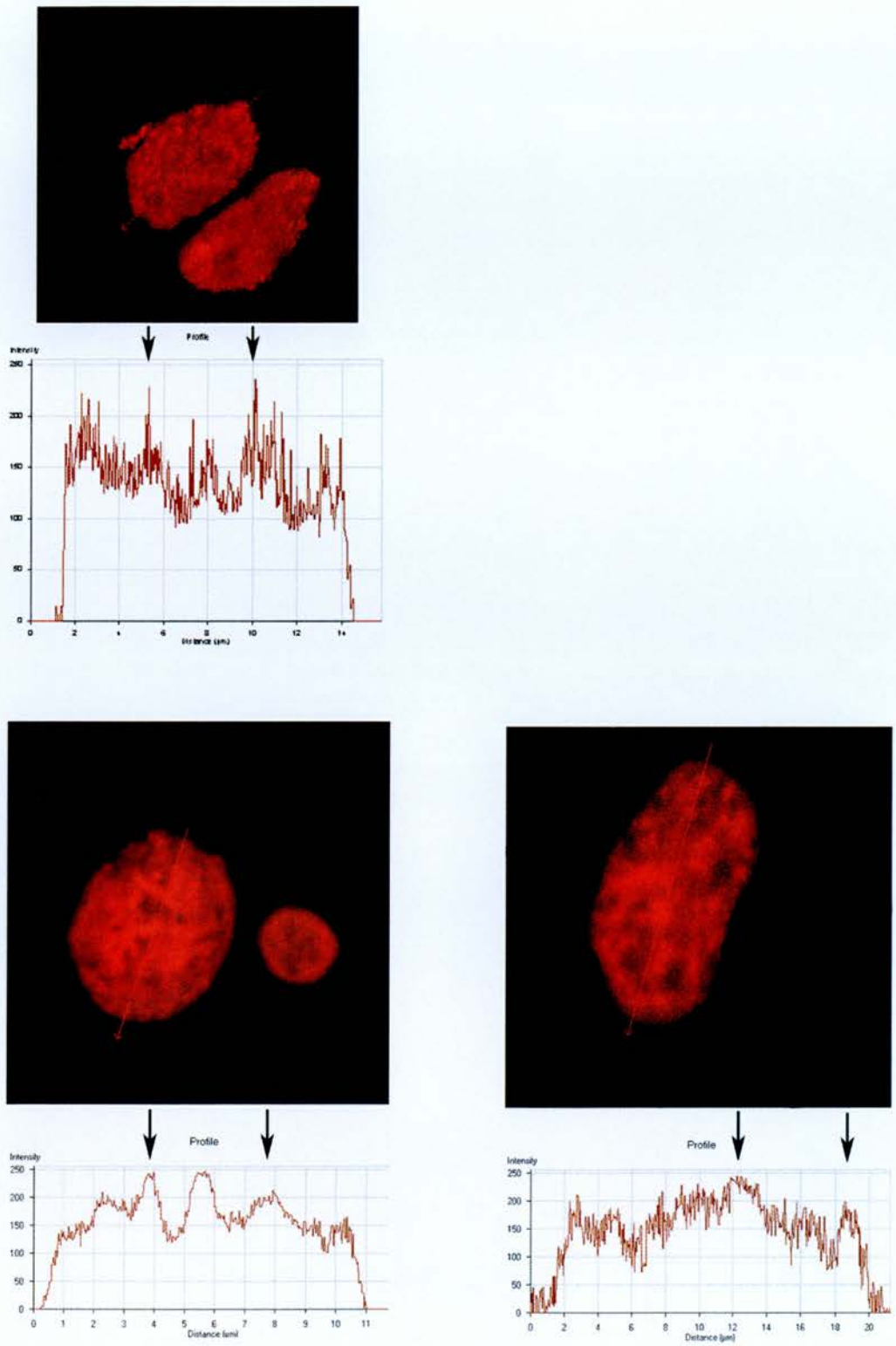


Figure 4.14b. ERα-DsRed expression in Hep G2 cells with addition of 10^{-8}M PPT™ for 60 minutes. Images are of three cell nuclei showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

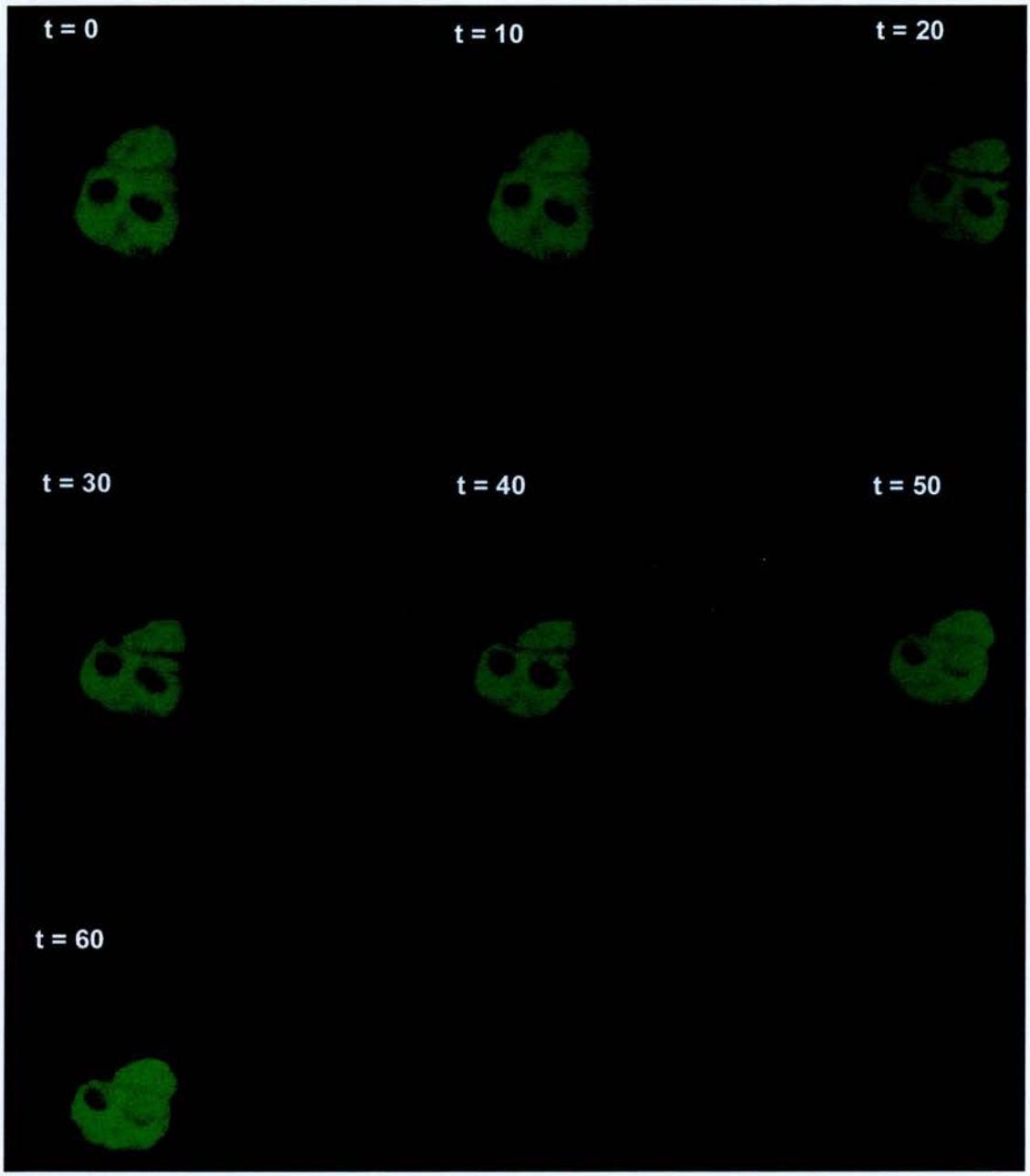


Figure 4.15a. ERβ1-EGFP expression in Hep G2 cells with addition of 10^{-8}M PPT™. The ERβ1-EGFP construct transiently transfected into Hep G2 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M PPT™. Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

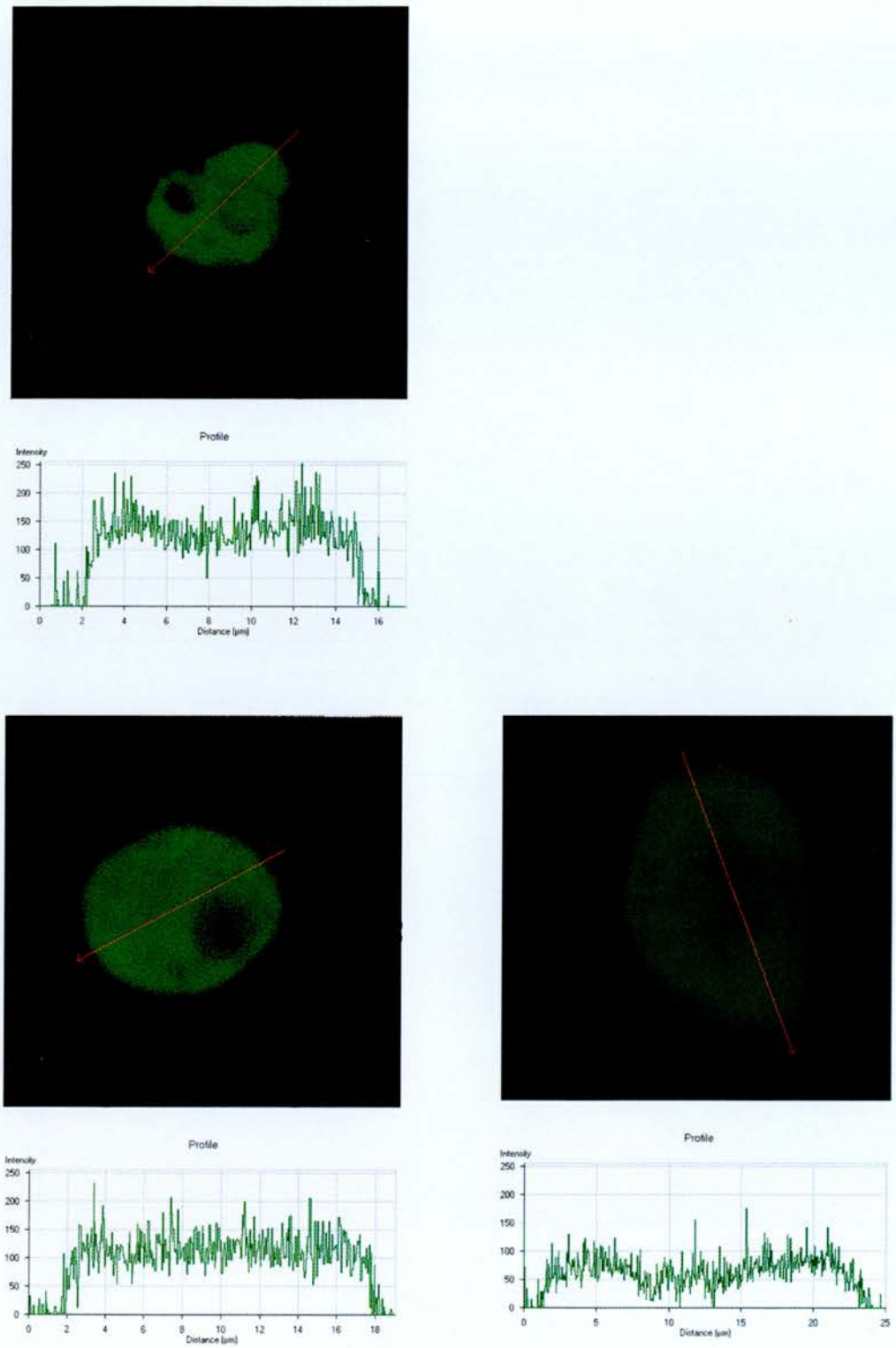


Figure 4.15b. ERβ1-EGFP expression in Hep G2 cells with addition of 10^{-8}M PPT™ for 60 minutes. Images are of three cell nuclei showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

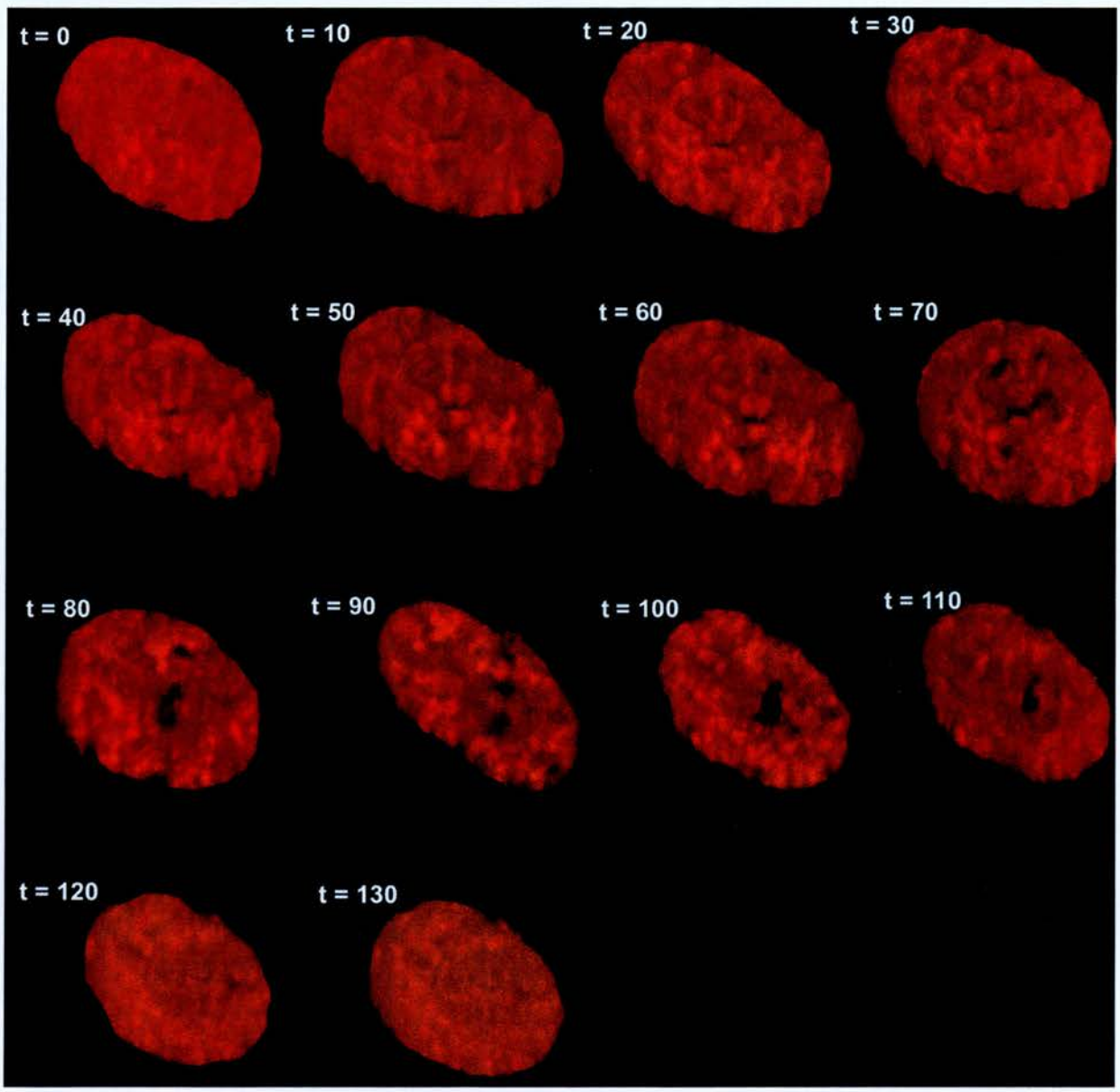


Figure 4.16a. ERα-DsRed expression in Hep G2 cells with addition of 10^{-8}M DPN™. The ERα-DsRed transiently transfected into Hep G2 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M DPN™. Confocal images taken over 130 minutes starting from time (t) 0, at 10 minute intervals.

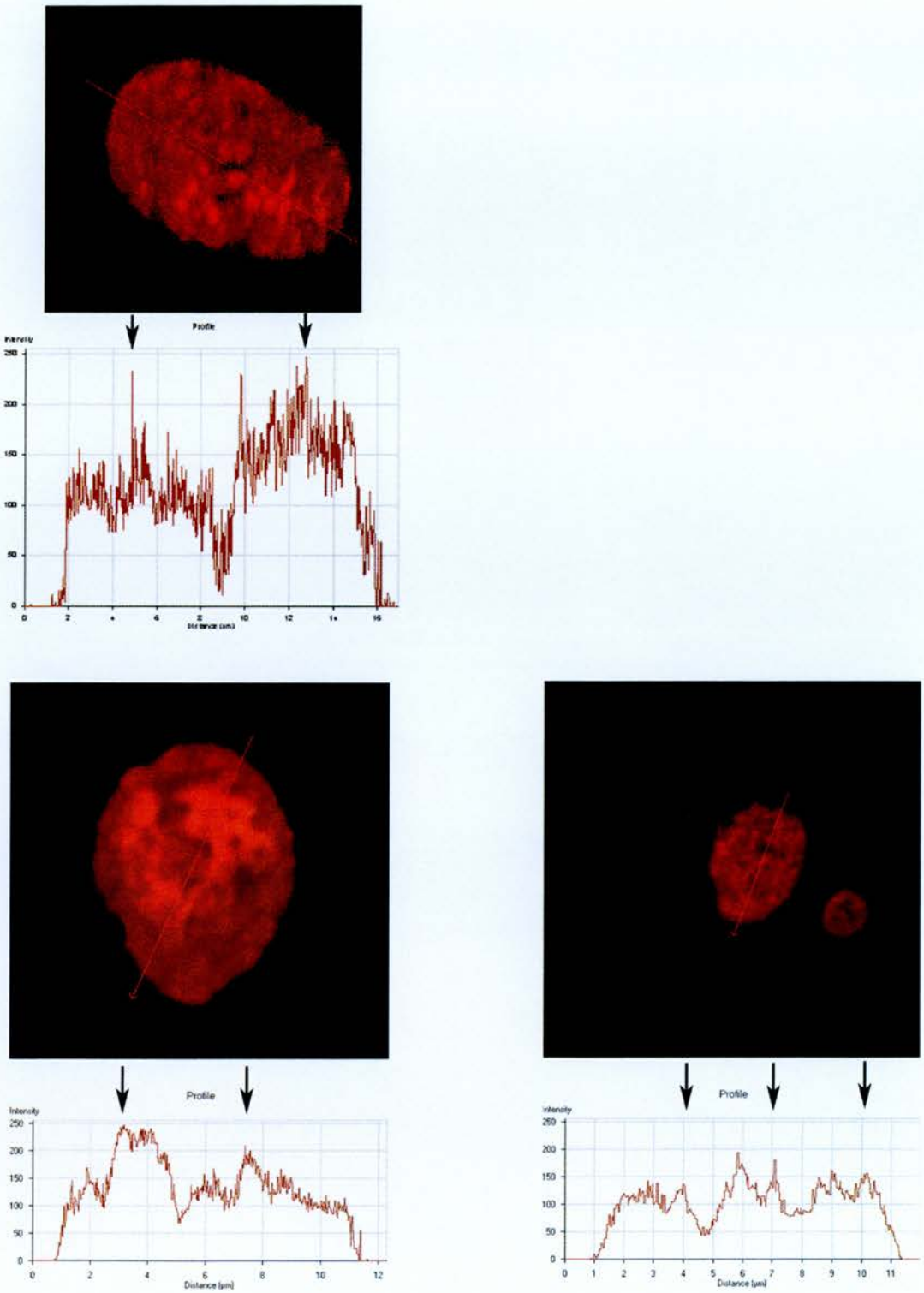


Figure 4.16b. ERα-DsRed expression in Hep G2 cells with addition of 10^{-8}M DPN™ for 60 minutes. Images are of three cell nuclei showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

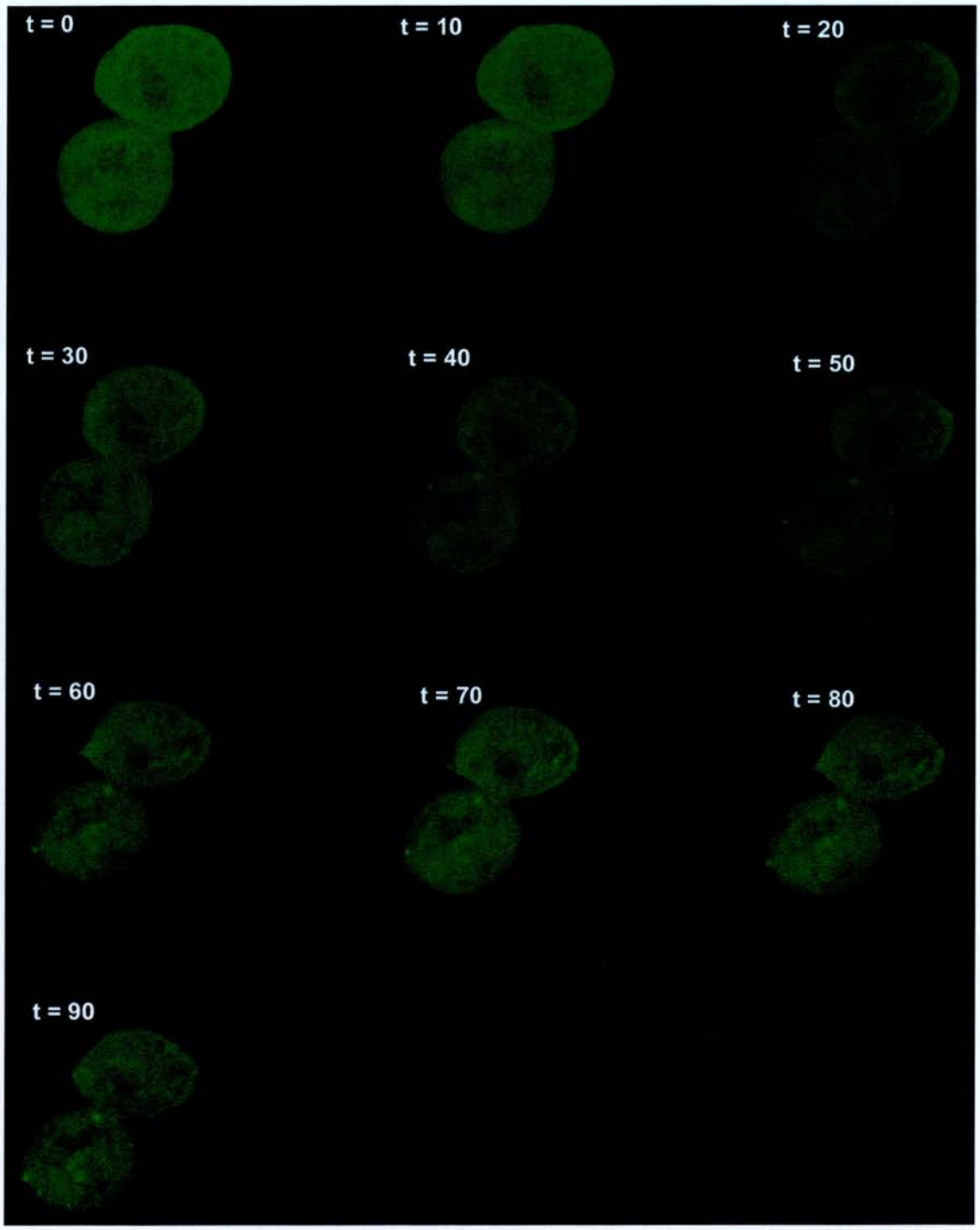


Figure 4.17a. ERβ1-EGFP expression in Hep G2 cells with addition of 10^{-8}M DPN™. The ERβ1-EGFP transiently transfected into Hep G2 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M DPN™. Confocal images taken over 90 minutes starting from time (t) 0, at 10 minute intervals.

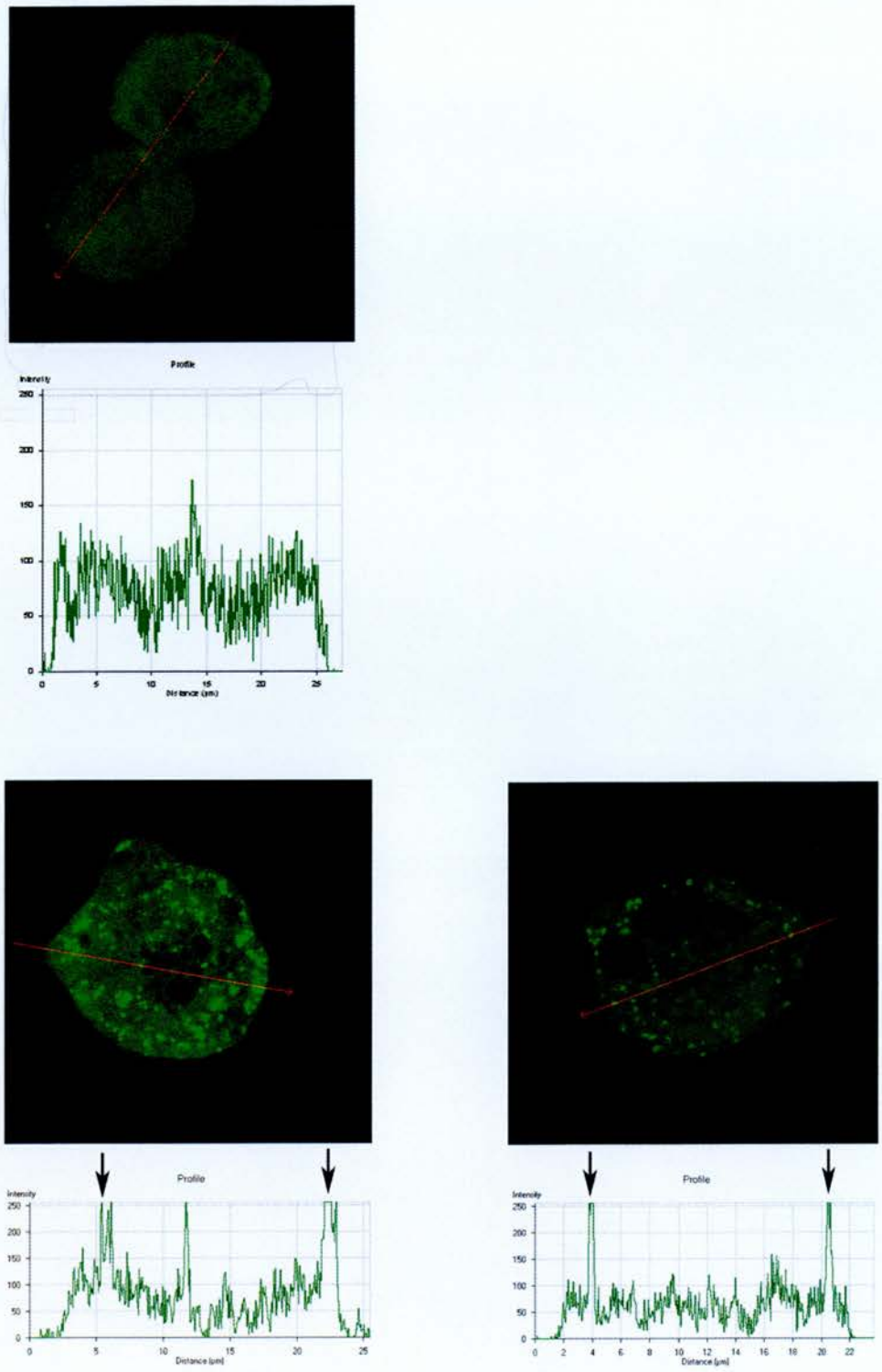


Figure 4.17b. ERβ1-EGFP expression in Hep G2 cells with addition of 10^{-8}M DPN™ for 60 minutes. Images are of three cell nuclei showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

4.3.7 Summary of ER distribution with Different Ligands

In the absence of ligand, ERα, ERβ1 and ERβ2 tagged to an FP were distributed in a uniform diffuse pattern within the nucleus. Upon addition of ligand ERα and ERβ1 redistributed to form bright foci, either as discrete foci or larger bright regions. ERβ2 failed to redistribute with E_2 due to its lack of an AF-2 region and therefore inability to bind ligands. The redistribution of the ERα-DsRed or ERβ1-EGFP upon addition of different ligands in Hek 293 and Hep G2 cells varied and is summarised in the tables below.

Table 4.1 Summary table for the ligand effect on the ER in Hek 293 cells.

Ligand	hERα-DsRed			hERβ1-EGFP		
(10^{-8}M)	Punctate?	Time	Max. difference	Punctate?	Time	Max. difference
E_2	Yes	10	40	Yes	10	60
3βAdiol	Yes	10	50	Yes	40	50

Time given in minutes.

Table 4.2. Summary table for the ligand effect on the ER in Hep G2 cells.

Ligand	hERα-DsRed			hERβ1-EGFP		
(10^{-8}M)	Punctate?	Time	Max. difference	Punctate?	Time	Max. difference
E_2	Yes	10	30	Yes	10	60
3βAdiol	Yes	10	30	Yes	40	80
Genistein	Yes	20	80	Yes	20	60
PPT™	Yes	10	50	No	-	-
DPN™	Yes	20	50	Yes	50	70

Time given in minutes.

4.3.8 Co-transfections of ERα, ERβ1 and ERβ2

4.3.8.1 Co-transfection of ERα and ERβ1

ERα-DsRed and ERβ1-EGFP were co-transfected into Hek 293 (*Figure 4.18a*) and Hep G2 (*Figure 4.19a*) cells. In some cells both ERα and ERβ1 were present in the same nuclei, however in other cells only one of the ERs was present (data not shown). ERα-DsRed and ERβ1-EGFP are represented by the colours of their FP tags, overlapping of the red and green FPs resulted in a yellow colour thus representing regions of co-expression of the two ER subtypes. In cells that had both ERs expressed the distribution of each receptor was altered by addition of ligand; ERα-DsRed redistributed into bright and darker regions with 10^{-8}M E_2 and ERβ1-EGFP formed more discrete foci however redistribution was not to the same extent as ERα. The intensity profiles (*Figures 4.18b* & *4.19b*) show that ERα-DsRed and ERβ1-EGFP did possibly heterodimerise, shown in yellow and formed foci together (arrows) however not on every occasion (*Figure 4.18b*). Formation of foci was more obvious in Hep G2 cells compared with Hek 293 cells. On some occasions it was easier to determine ERα-DsRed and ERβ1-EGFP co-localisation from the nuclear image rather than the intensity profiles (*Figure 4.19b*).

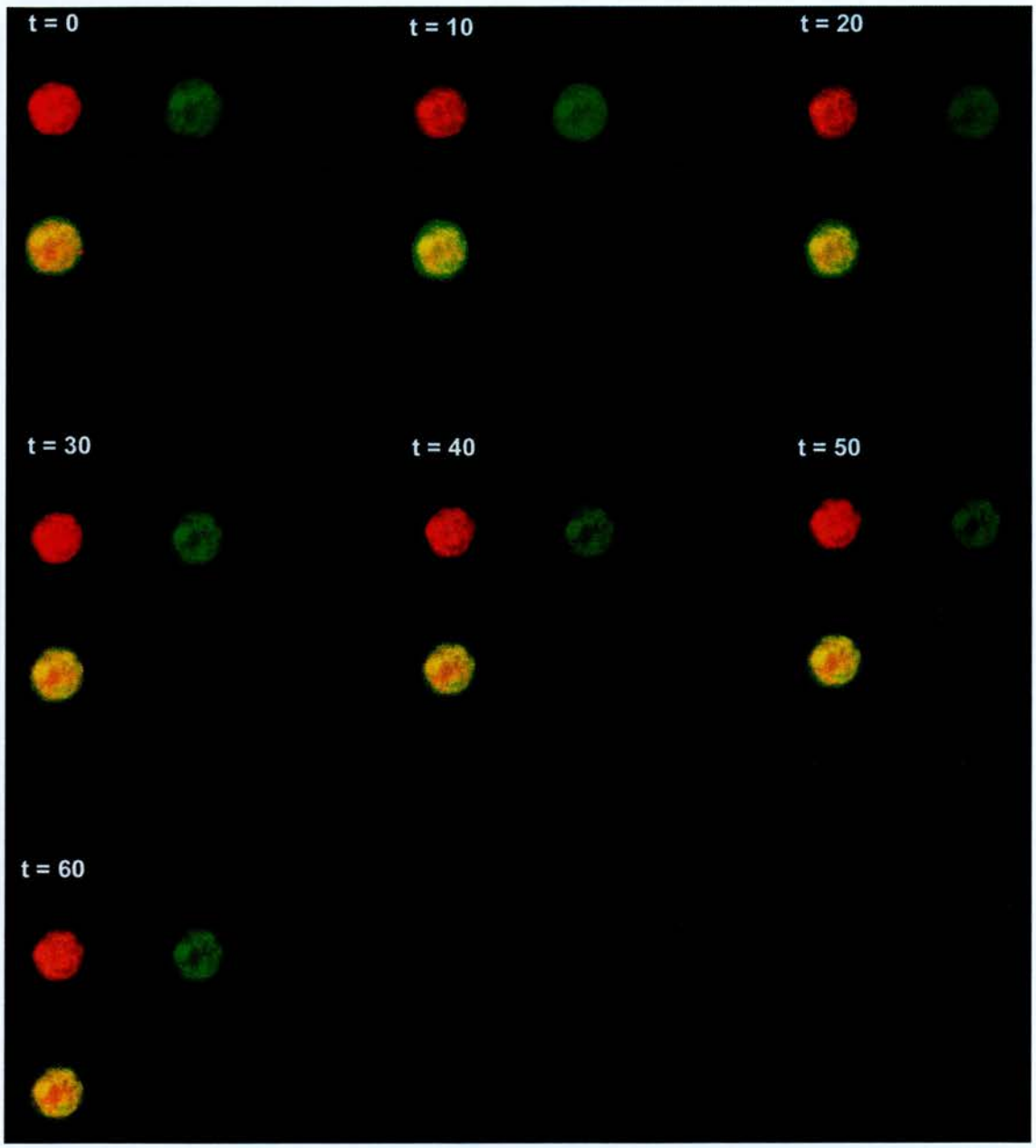


Figure 4.18a. ERα-DsRed and ERβ1-EGFP co-expression in Hek 293 cells with addition of 10^{-8}M E_2. ERα-DsRed and ERβ1-EGFP transiently transfected into Hek 293 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M E_2. Each time point shows the red (ERα-DsRed) channel, the green channel (ERβ1-EGFP) and both channels together (yellow). Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

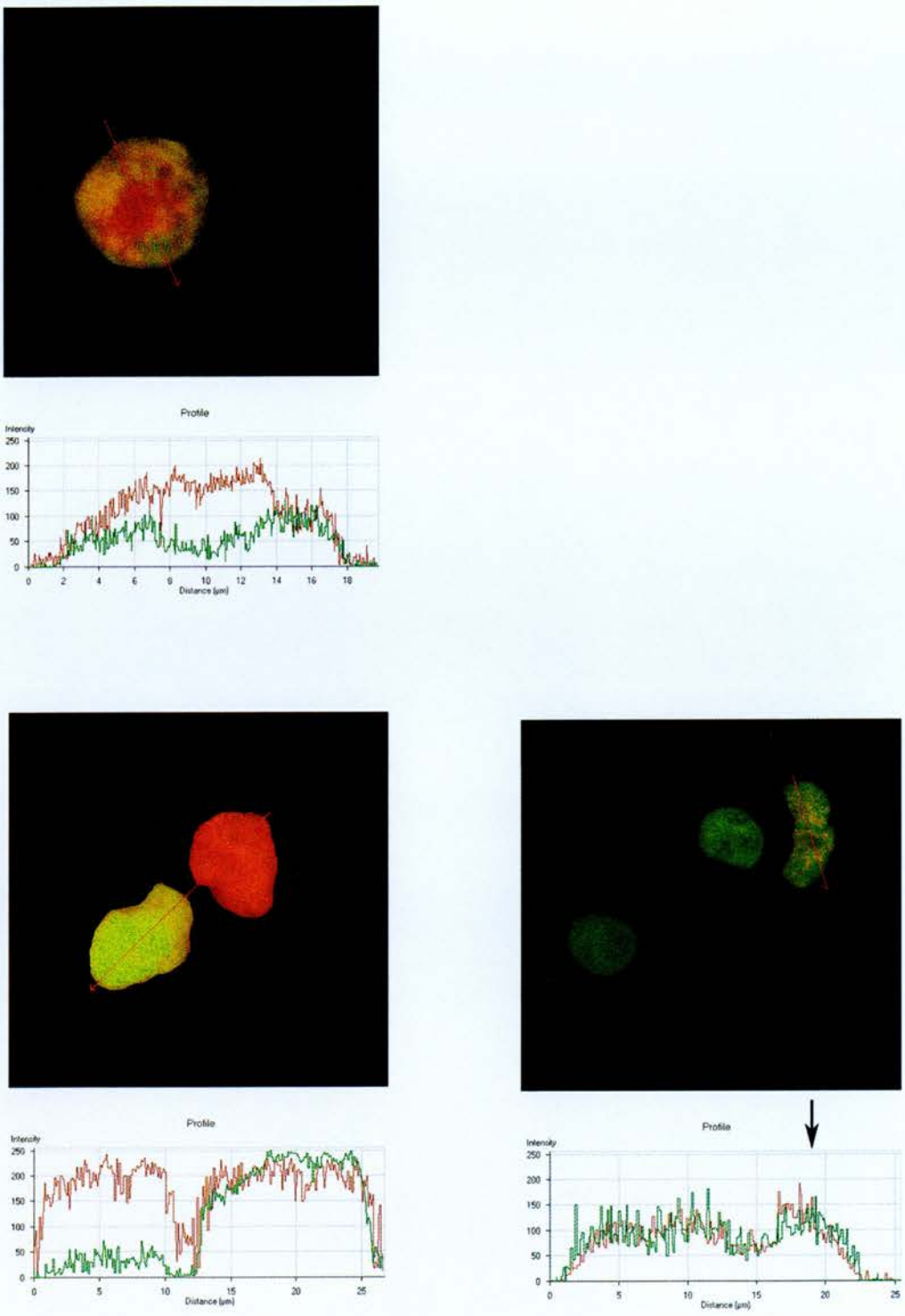


Figure 4.18b. ERα-DsRed and ERβ1-EGFP co-expression in Hek 293 cells with addition of 10^{-8}M E_2 for 60 minutes. Images are of three cell nuclei (red and green channels together) showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

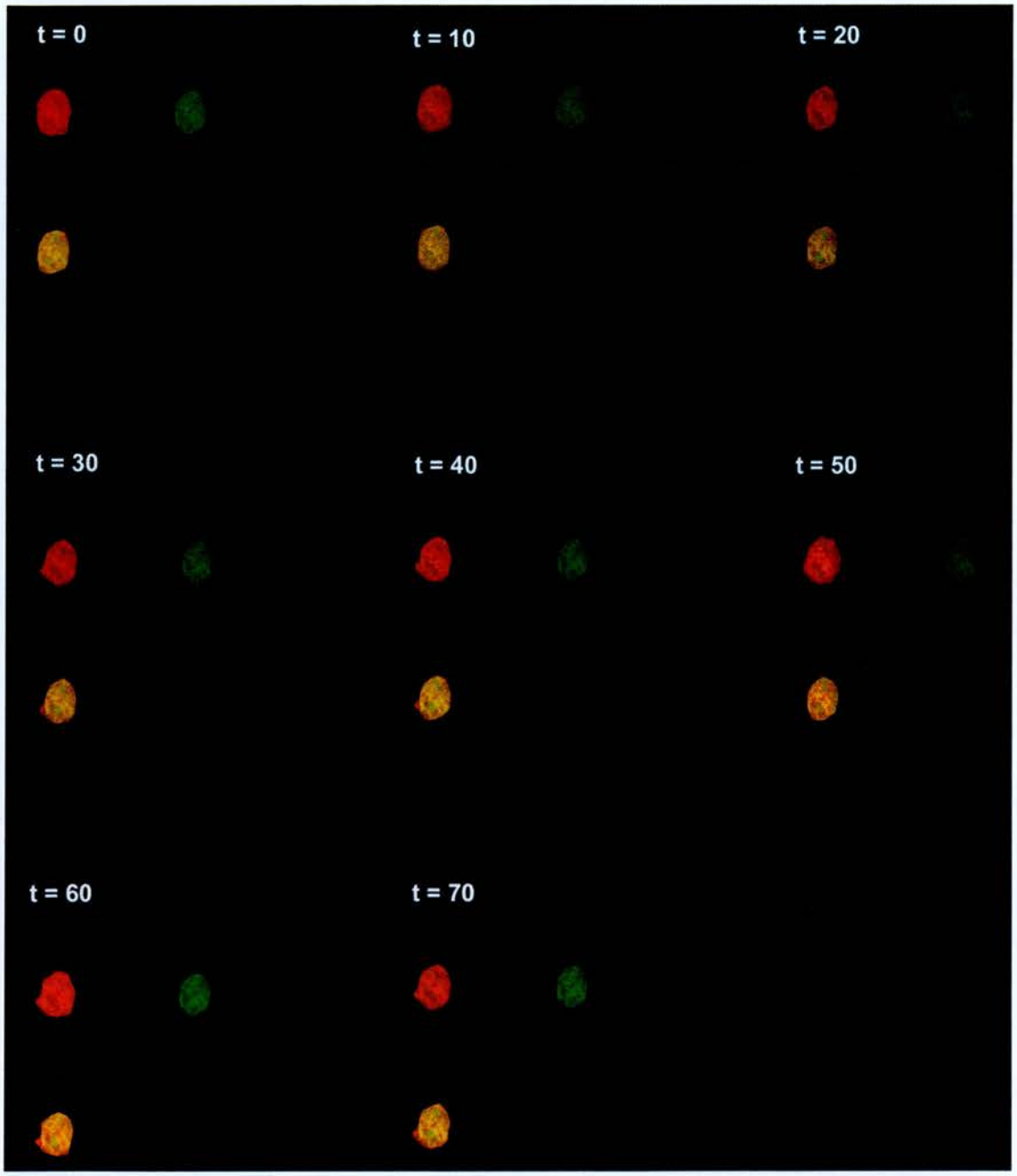


Figure 4.19a. ERα-DsRed and ERβ1-EGFP co-expression in Hep G2 cells with addition of 10^{-8}M E_2. ERα-DsRed and ERβ1-EGFP transiently transfected into Hep G2 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M E_2. Each time point shows the red (ERα-DsRed) channel, the green channel (ERβ1-EGFP) and both channels together (yellow). Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

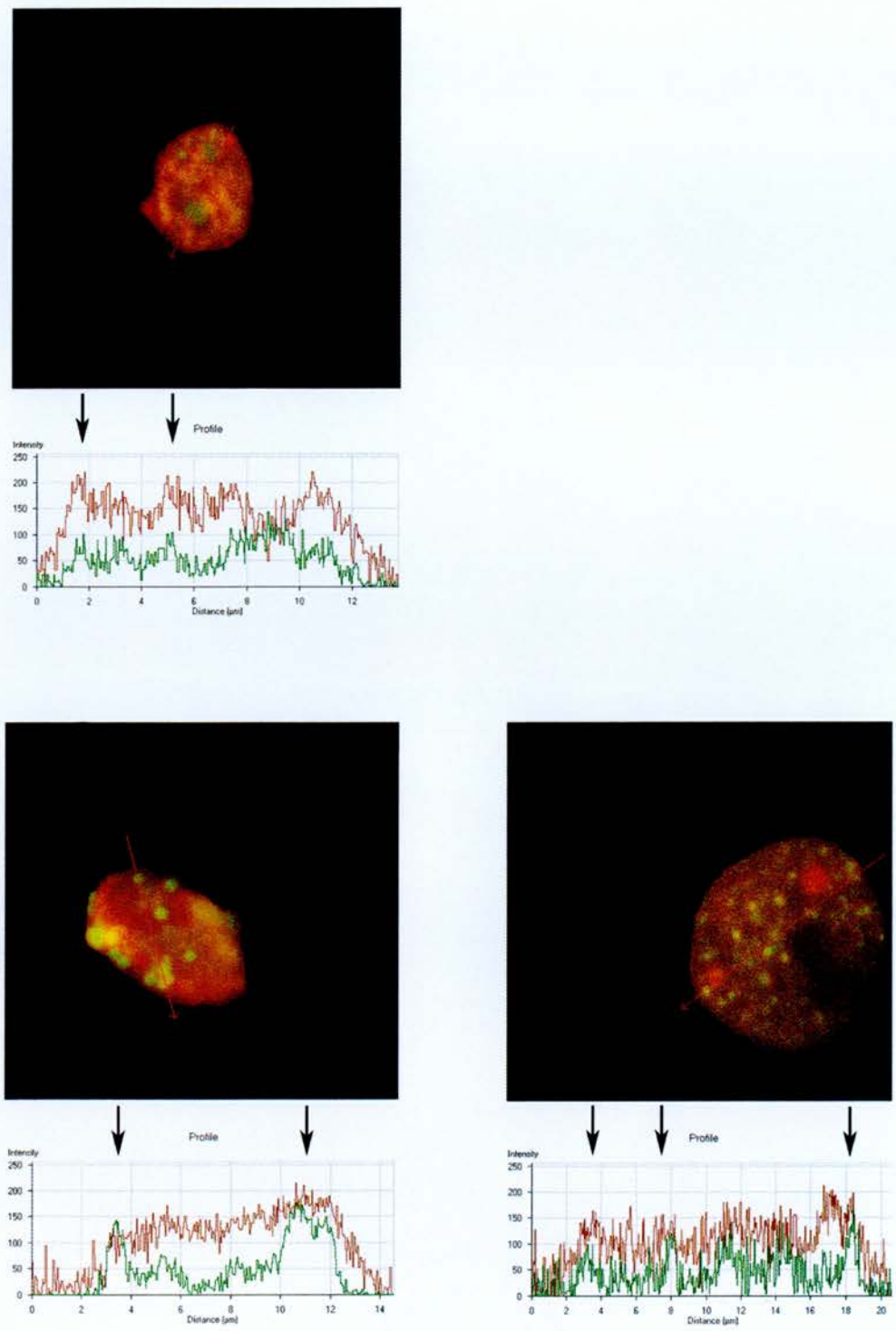


Figure 4.19b. ERα-DsRed and ERβ1-EGFP co-expression in Hep G2 cells with addition of 10^{-8}M E_2 for 60 minutes. Images are of three cell nuclei (red and green channels together) showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

4.3.8.2 Co-transfection of ERα and ERβ2

ERα-DsRed and ERβ2-EGFP were co-transfected into Hek 293 (Figure 4.20a) and Hep G2 (Figure 4.21a) cells. Both of these receptors were diffusely distributed within the same nucleus of some cells, indicated in yellow, and their distribution altered upon addition of 10^{-8}M E_2. In Hek 293 cells (Figure 4.20a) ERα-DsRed relocated to form a focal distribution after 10 minutes with 10^{-8}M E_2, however ERβ2-EGFP remained in a diffuse distribution. The intensity profiles (Figure 4.20b) demonstrate that ERβ2-EGFP remained in a fairly uniform distribution with a couple of peaks in fluorescence possibly indicating ERβ2-EGFP formed heterodimers with ERα-DsRed, appearing as yellow foci. In one of the intensity profiles there was a green peak without a red peak, possibly indicating ERβ2/ERβ2 homodimers occurred, although ERβ2 lacks the functional AF-2 domain.

In Hep G2 cells (Figure 4.21a) the ERα-DsRed redistributed into foci after 10 minutes with 10^{-8}M E_2, ERβ2-EGFP distribution also altered. ERβ2-EGFP changed from a diffuse distribution to darker and lighter green regions rather than specific observable foci. The intensity profiles (Figure 4.21b) show in different cells the redistribution of ERβ2-EGFP differs and that it can form foci with ERα-DsRed (shown in yellow) and therefore heterodimers may have formed. The intensity profiles show clear peaks and troughs with ERα-DsRed and ERβ2-EGFP, some of which appear coincidental (arrows), although they did not appear as a very distinct yellow (red/green) overlap when viewed under the confocal.

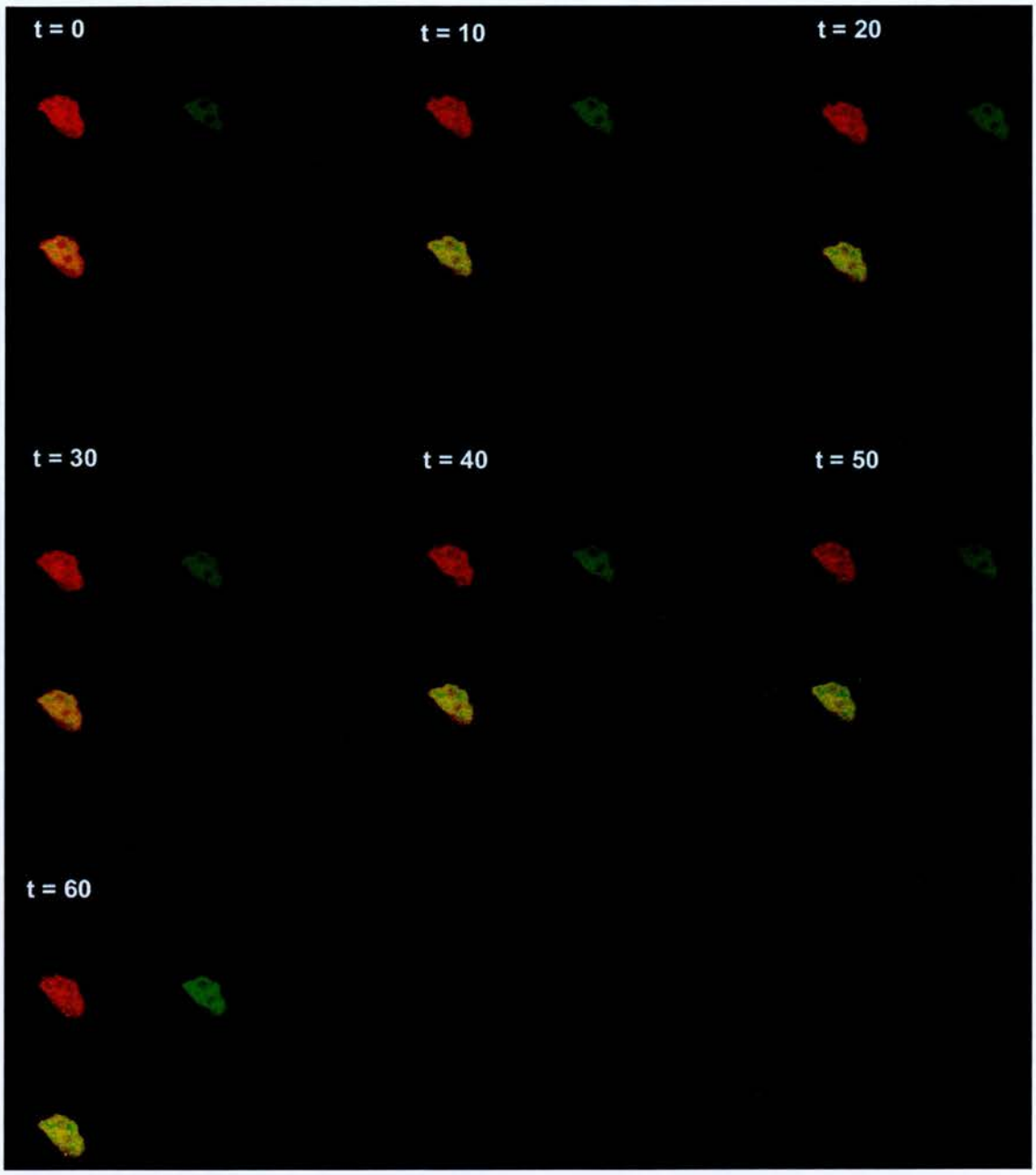


Figure 4.20a. ERα-DsRed and ERβ2-EGFP co-expression in Hek 293 cells with addition of 10^{-8}M E_2. ERα-DsRed and ERβ2-EGFP transiently transfected into Hek 293 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M E_2. Each time point shows the red (ERα-DsRed) channel, the green channel (ERβ2-EGFP) and both channels together (yellow). Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

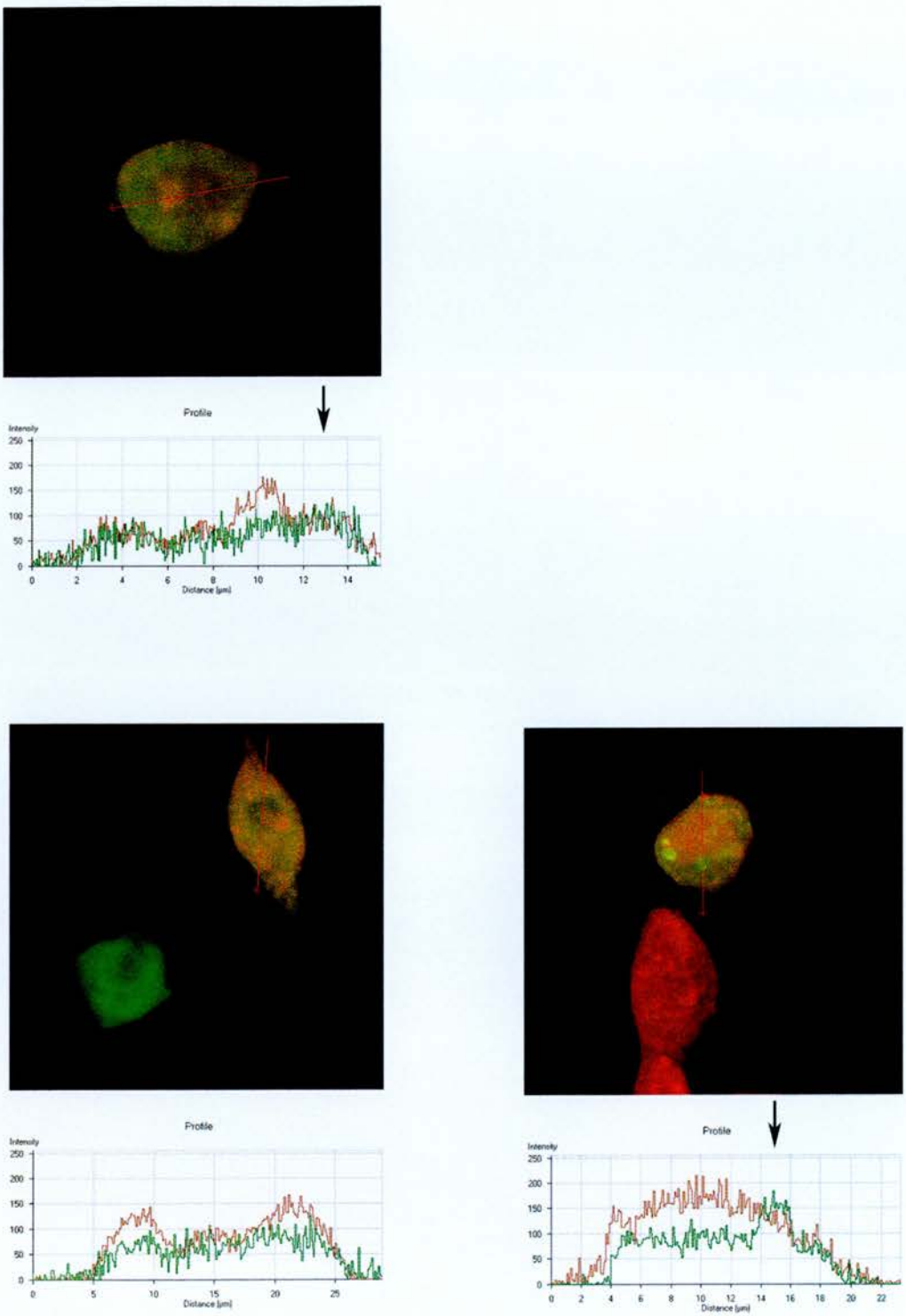


Figure 4.20b. ERα-DsRed and ERβ2-EGFP co-expression in Hek 293 cells with addition of 10^{-8}M E_2 for 60 minutes. Images are of three cell nuclei (red and green channels together) showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

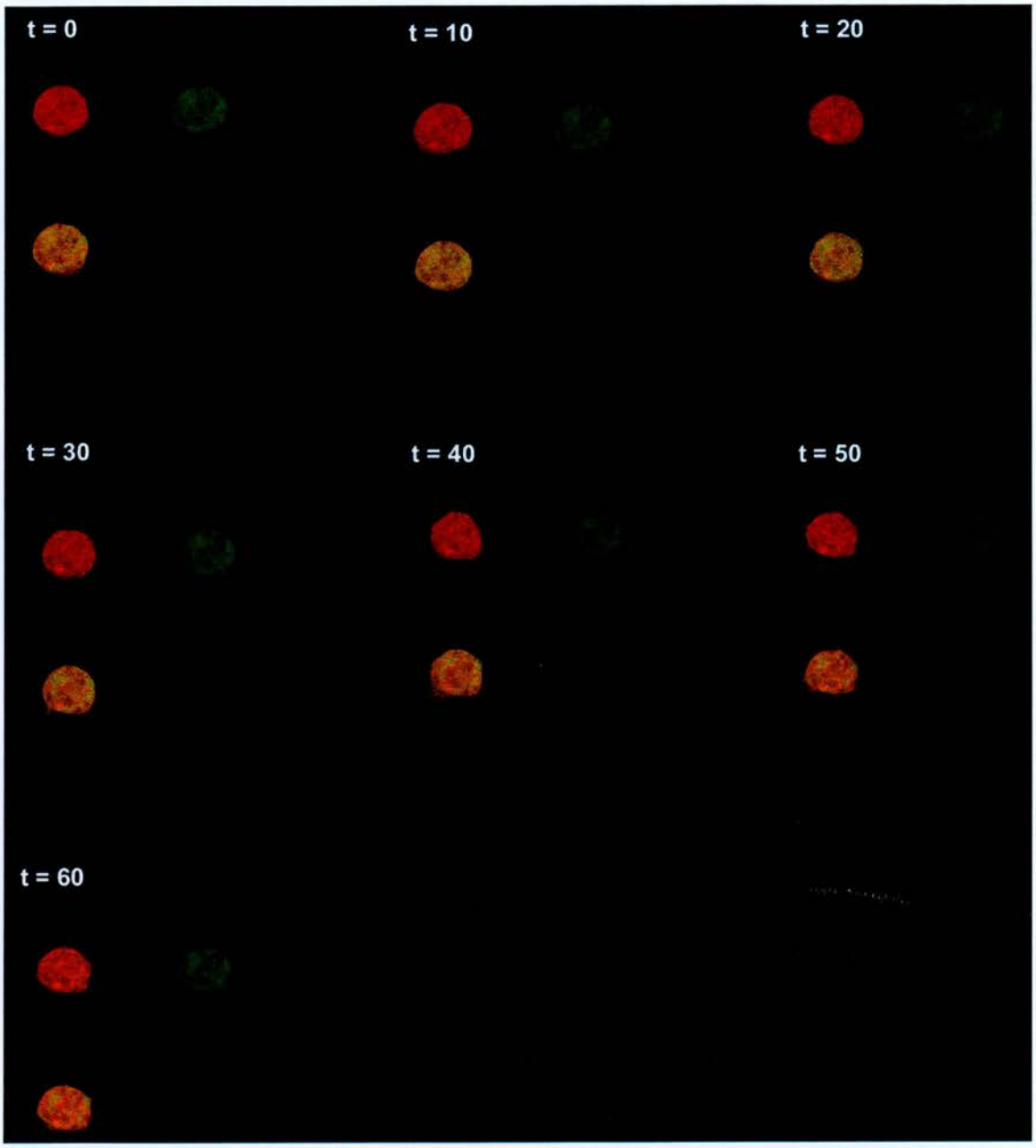


Figure 4.21a. ERα-DsRed and ERβ2-EGFP co-expression in Hep G2 cells with addition of 10^{-8}M E_2. ERα-DsRed and ERβ2-EGFP transiently transfected into Hep G2 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M E_2. Each time point shows the red (ERα-DsRed) channel, the green channel (ERβ2-EGFP) and both channels together (yellow). Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

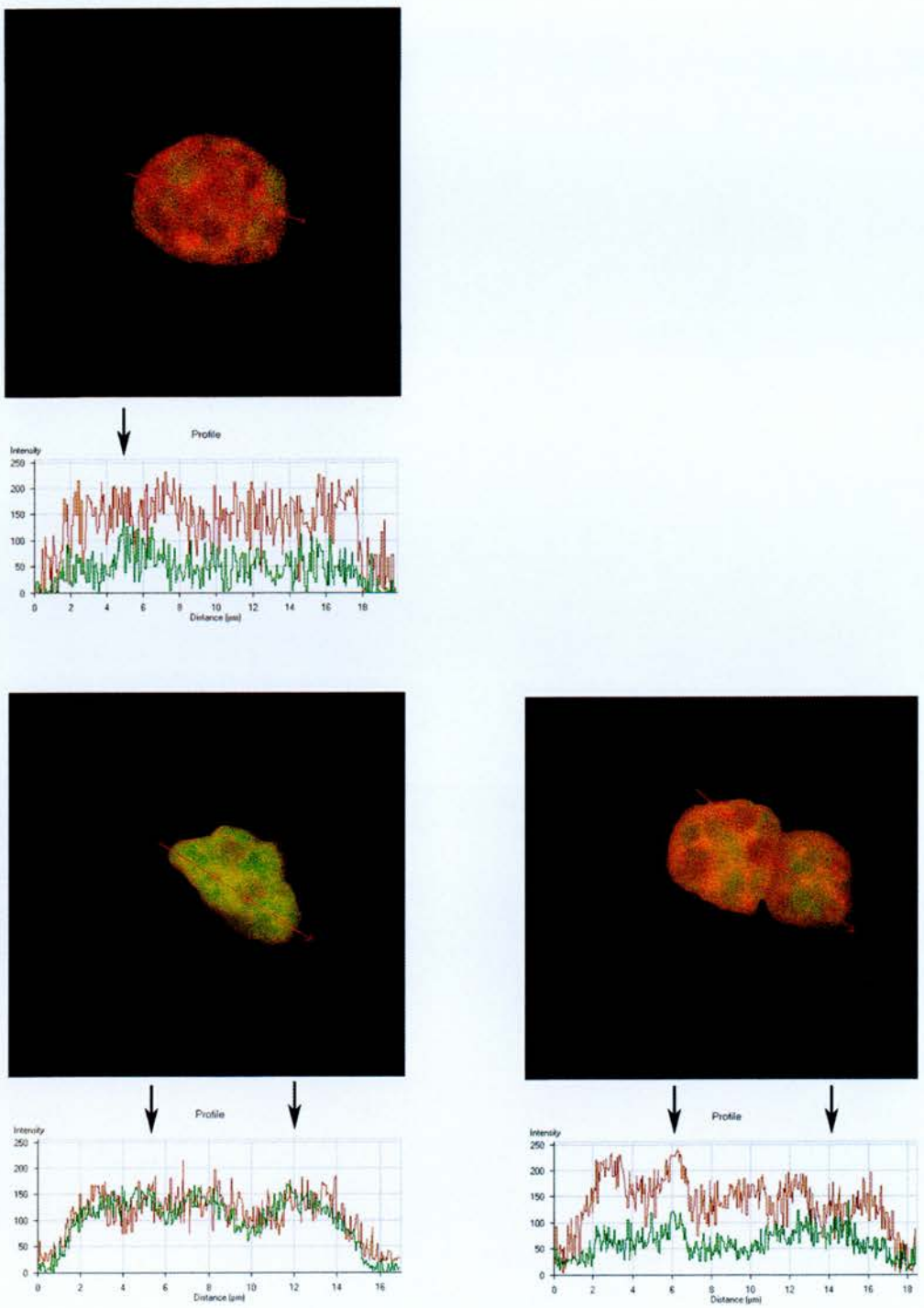


Figure 4.21b. ERα-DsRed and ERβ2-EGFP co-expression in Hep G2 cells with addition of 10^{-8}M E_2 for 60 minutes. Images are of three cell nuclei (red and green channels together) showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

4.3.8.3 Co-transfection of ERβ1 and ERβ2

ERβ1-DsRed and ERβ2-EGFP were co-transfected in Hek 293 (*Figure 4.22a*) and Hep G2 (*Figure 4.23a*) cells. These two receptors were present in the same nuclei, and responded to 10^{-8}M E_2. ERβ1-DsRed redistributed to a focal pattern in both cell lines, however ERβ2-EGFP only redistributed to a noticeably focal pattern in the Hep G2 cells (*Figure 4.23a*) forming foci in the same areas as the ERβ1-DsRed foci. The intensity profiles for the Hek 293 (*Figure 4.22b*) demonstrate that the two receptors show a similar distribution with possible focal regions of both ERs together (yellow areas). The peaks of intensity of ERβ1-DsRed were consistent with the peaks of ERβ2-EGFP (arrows) although discrete foci were not visible. In Hep G2 cells (*Figure 4.23b*) the intensity profiles for ERβ1-DsRed and ERβ2-EGFP were similar and it could be observed that ERβ1-DsRed and ERβ2-EGFP may have co-localised in the same foci within the nucleus as shown in the intensity profile (arrows).

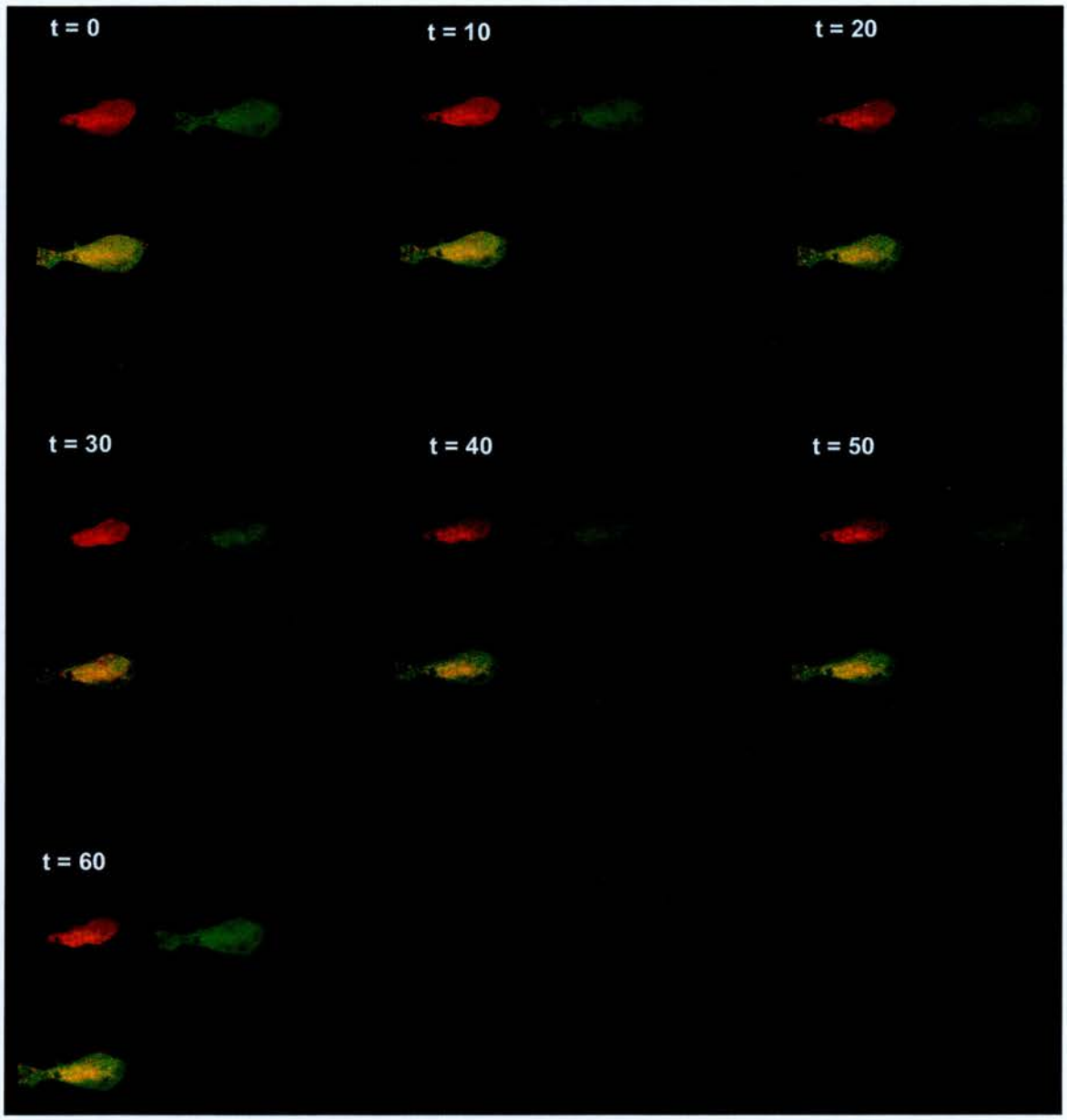


Figure 4.22a. ERβ1-DsRed and ERβ2-EGFP co-expression in Hek 293 cells with addition of 10^{-8}M E_2. ERβ1-DsRed and ERβ2-EGFP transiently transfected into Hek 293 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M E_2. Each time point shows the red (ERβ1-DsRed) channel, the green channel (ERβ-EGFP) and both channels together (yellow). Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

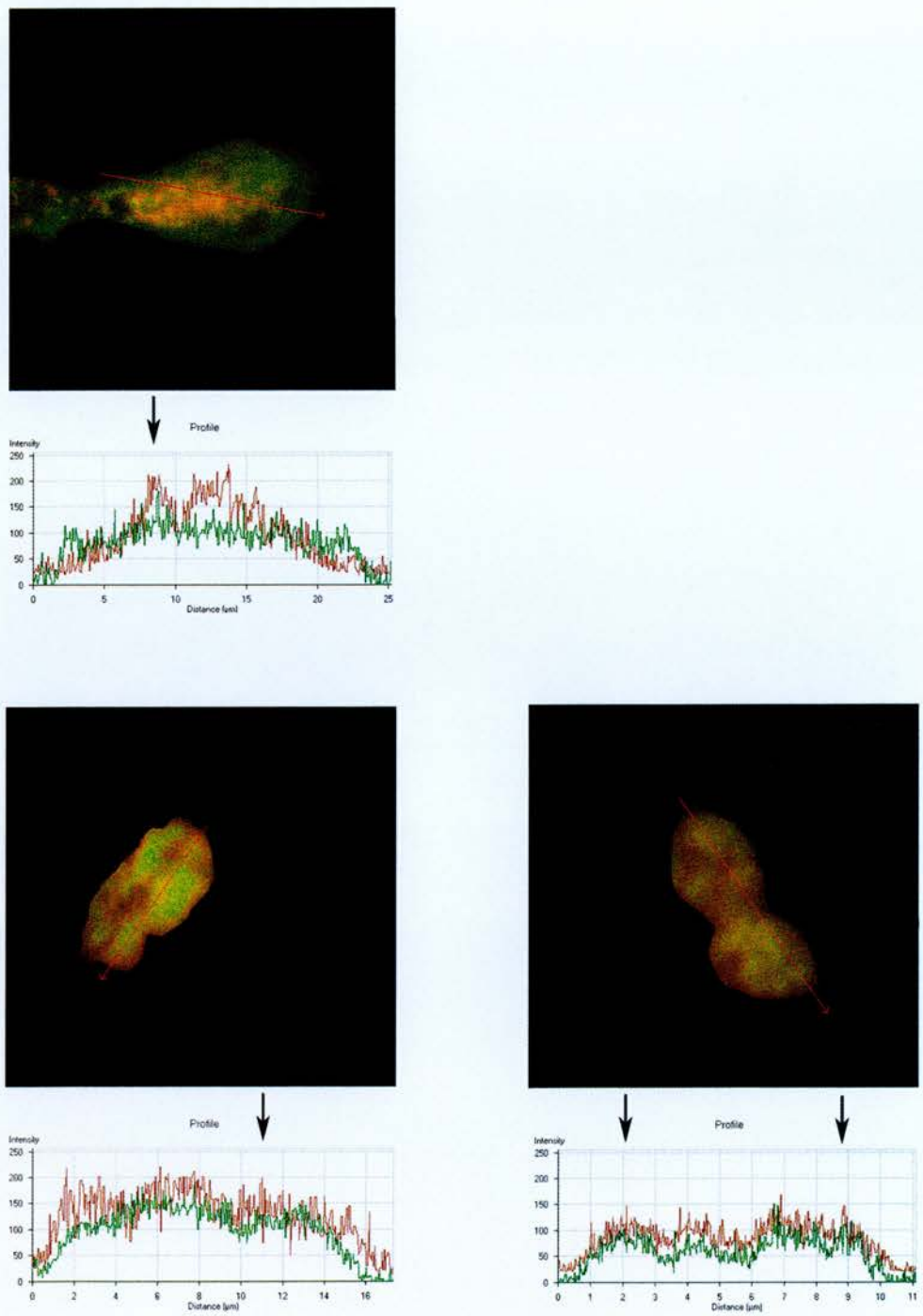


Figure 4.22b. ERβ1-DsRed and ERβ2-EGFP co-expression in Hek 293 cells with addition of 10^{-8}M E_2 for 60 minutes. Images are of three cell nuclei (red and green channels together) showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

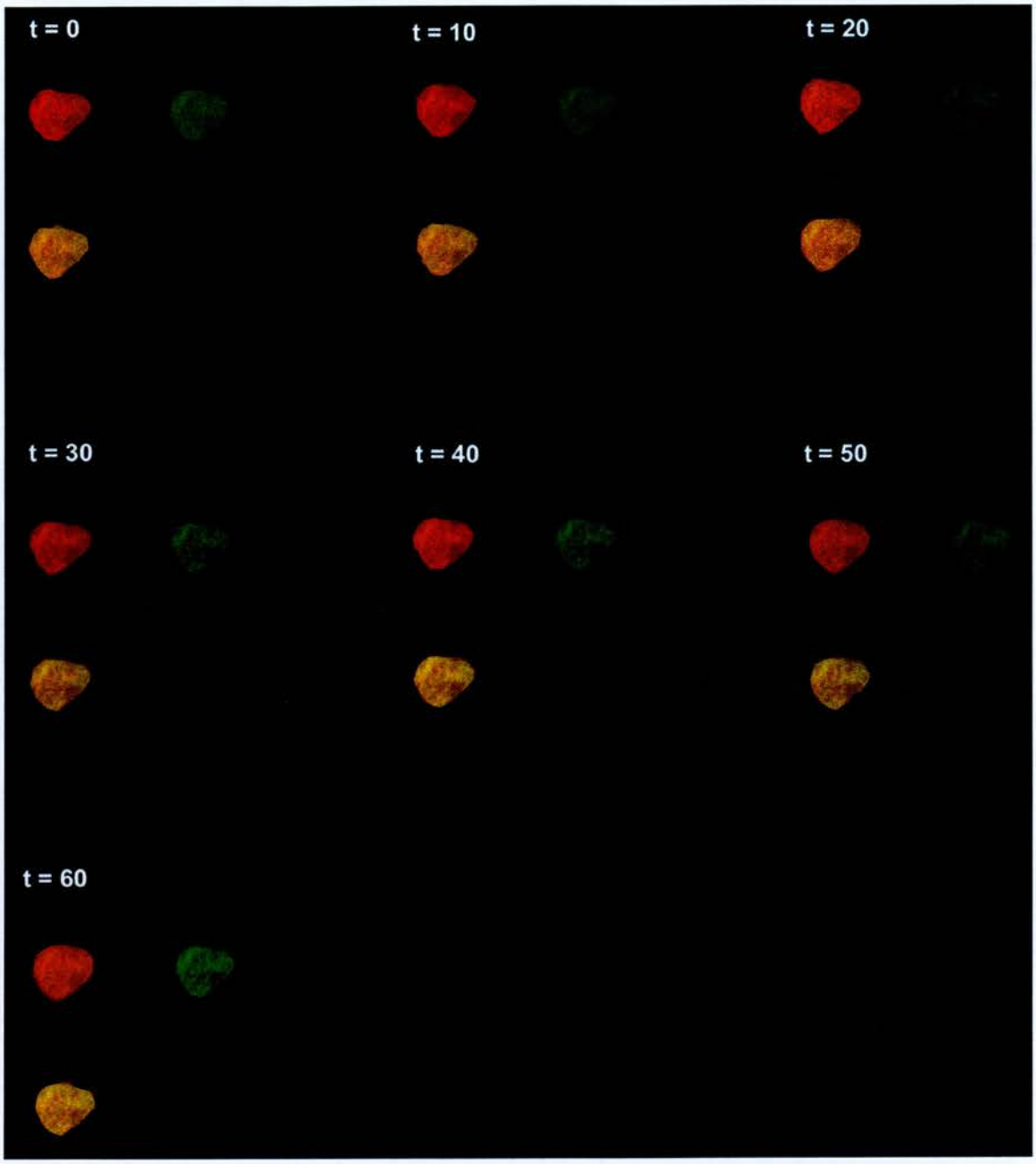


Figure 4.23a. ERβ1-DsRed and ERβ2-EGFP co-expression in Hep G2 cells with addition of 10^{-8}M E_2. ERβ1-DsRed and ERβ2-EGFP transiently transfected into Hep G2 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M E_2. Each time point shows the red (ERβ1-DsRed) channel, the green channel (ERβ2-EGFP) and both channels together (yellow). Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

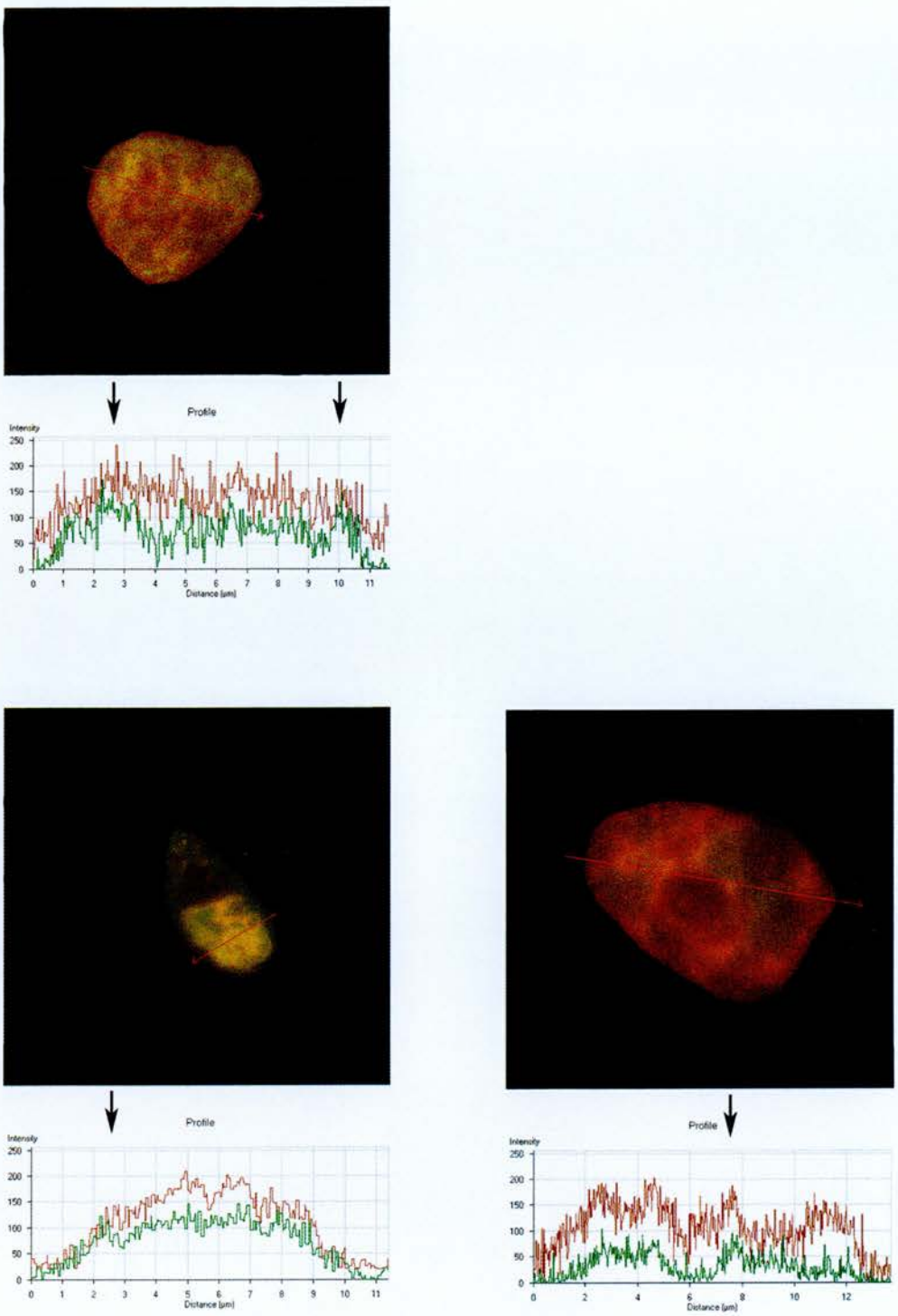


Figure 4.23b. ERβ1-DsRed and ERβ2-EGFP co-expression in Hep G2 cells with addition of 10^{-8}M E_2 for 60 minutes. Images are of three cell nuclei (red and green channels together) showing a graph of the intensity of the fluorescence along the red line drawn through the nucleus.

4.4 Discussion

These results have demonstrated that human ERα and ERβ1 proteins tagged at their N-terminal using a fluorescent protein have a diffuse distribution within the cell nucleus and that upon addition of an oestrogenic ligand, the receptor redistributes into bright foci. Similar findings have been reported previously by several laboratories following studies using FP-hERα and E_2 (Htun *et al.*, 1999; Stenoien *et al.*, 2000; Stenoien *et al.*, 2001a; Weatherman *et al.*, 2002). And a single report has investigated the behaviour of FP-hERβ1 (Matsuda *et al.*, 2002).

Attempts at defining the two biologically distinct states of ER by immunocytology have failed to reveal any differences in the localisation of the loosely and tightly bound forms (Greene and Press, 1986; Yamashita, 1995). The advent of FP-tagged receptors and high resolution microscopy has allowed FP-ER localisation and redistribution to be examined in live cells. This is advantageous as it avoids the artefacts caused by fixation methods or antibody labelling. For the first time it has been possible to observe the differences in intranuclear distribution of the receptor that reflected the type of ligand, agonist or antagonist, bound to the receptor. Htun *et al*'s work represented the first report of the importance of ligand in affecting the distribution of a nuclear receptor within the nucleus (Htun *et al.*, 1996).

The EGFP protein is 27 kDa and the DsRed protein is 28 kDa, it was originally thought that these proteins may have interfered with the function and localisation of the tagged ER, however this has not been reported. When an ER is tagged to an FP the receptor remains functional within the nucleus and it is capable of transcriptional activation of the ERE-reporter gene upon addition of E_2 with similar activity to that of the untagged ER (Htun *et al.*, 1999; Stenoien *et al.*, 2000; Weatherman *et al.*, 2002). During the course of the current investigations, fluorescently tagged ERα and ERβ1 activated an ERE-Luc reporter when they were incubated with E_2. The observed dose response was comparable to the fold increases observed with the untagged ER constructs (Chapter 5) (FP data not shown). Western analysis using proteins extracted from cells transiently transfected with the FP tagged-ER constructs have shown the ER proteins are expressed and that their apparent molecular weight is consistent with the expected size of the full length ER plus the FP tag. Therefore it can be assumed that the FP tag does not disrupt the expression of a functional ER

within a cell, or the receptor's ability to activate gene expression via an oestrogen response element.

In the current series of experiments differences in the subcellular localisation of ERα, ERβ1 and ERβ2 in the different cell types were observed following exposure to ligand. Furthermore, the pattern of intranuclear redistribution was influenced by the ligand used. In the absence of ligand ERα and ERβ1 proteins tagged to the FP were mostly distributed in a diffuse manner throughout the nucleus (but not observable in the nucleolus of some cells). A few brighter "foci" were also observed in the absence of ligand, this may have been due to active redistribution initiated by endogenous ligand or co-activators. This was more apparent with ERα-DsRed than with the ERβ1-EGFP protein.

Htun *et al* were the first to report the redistribution of hERα-GFP in the presence of E_2, from a reticular (network) pattern evenly distributed throughout the nucleus, to a focal or punctate pattern (Htun *et al.*, 1999). In the current studies both FP-hERα and FP-hERβ1 altered their nuclear distribution following addition of ligand with discrete foci appearing. ERα-DsRed redistributed with all of the ligands tested including E_2, 3βAdiol and the synthetic PPT™. The commercial agonist DPN™ sold as a more potent activator of ERβ1 than ERα also activated ERα-DsRed, as did genistein which has also been shown to be a more potent activator of ERβ1 than ERα (An *et al.*, 2001). ERβ1-EGFP was activated to redistribute into punctate foci by all the ligands except the ERα-specific ligand PPT™. The redistribution of ERβ2-EGFP was not altered by exposure to E_2 and the FP tagged protein remained in a diffuse distribution. Upon addition of ligand ERβ1-EGFP formed more discrete foci compared to the larger foci of ERα-DsRed. This was more apparent in the Hek 293 cells compared to the Hep G2 cells. This difference in focal pattern may be due to the DsRed protein tag or the ERα construct, therefore more work is required using the ERβ1-DsRed construct to determine whether the observed effect is due to the different tags or due to the different ER subtypes.

FP-ERα has been shown to activate a reporter gene in a dose dependent manner (Htun *et al.*, 1999). In those studies maximal activation of ERα-GFP by E_2 was observed using a concentration of 10nM (10^{-8}M). Increasing the concentration of ligand from 1nM to 100nM has been reported to decrease the time for an ER-GFP

to redistribute into a speckled pattern (Price *et al.*, 2001). In the experiments presented here, 10^{-8}M of each ligand was used as this was found to be the optimum concentration required to stimulate ERα and ERβ1 induced reporter gene activation using an ERE-Luc reporter (Chapter 5).

It has previously been demonstrated that the ER associates with the nuclear matrix (Htun *et al.*, 1999). However, Stenoien *et al* demonstrated using fluorescent recovery after photobleaching (FRAP) that nuclear receptors, whether bound to chromatin or any other type of nuclear structure, are much more dynamic than previously thought (Stenoien *et al.*, 2001b). The rapid exchange rates observed with these receptor proteins has led to conflicting interpretations of the mechanism of intranuclear mobility. Agonist bound ERs form nuclear matrix bound intranuclear foci, the individual receptors within these foci are able to undergo rapid exchange as observed using FRAP. However, another report suggested that a reduced mobility of the nuclear proteins upon ligand binding and redistribution is thought to be due to interactions with immobilised components of the nuclear architecture (McKenna *et al.*, 1998). The size of the receptor complexes does not significantly change when ligand is added, therefore indicating the observed reduction in mobility is not due to the increased molecular interactions that reduce brownian motion, but rather the reduction in mobility is probably due to interaction with a nuclear substrate. Stenoien *et al* have demonstrated that ERα-GFP can exhibit different rates of mobility upon addition of different agonists or antagonists due to conformational alterations. For example the antioestrogen ICI 182,780 renders ERα-GFP immobile, even if the cell has been treated with E_2 (Stenoien *et al.*, 2001a).

However it has been demonstrated, using Br-UTP and/or antibodies to RNA polymerase II, that that there is less co-localisation between RNA synthesis foci and transcription factors than previously expected (Stenoien *et al.*, 2000). High resolution microscopic analysis has revealed that most FP-hERα foci do not overlap with transcription sites (Stenoien *et al.*, 2000). These data suggest that complexes containing transcription factors are in a dynamic state of assembly/disassembly and that the time spent actively transcribing genes may be minimal.

Several laboratories have already demonstrated that FP-ERα develops a focal distribution upon addition of E_2 (Htun *et al.*, 1999; Stenoien *et al.*, 2000; Stenoien *et al.*, 2001a; Weatherman *et al.*, 2002). By following individual cells after addition of

ligand they demonstrated that re-organisation occurs on a rapid timescale of 10 to 20 minutes. In the current studies the time it took for the ERα-DsRed to redistribute into a focal pattern following addition 10^{-8}M E_2 was also 10 minutes, however the time scale differed using other ligands. In general ERα-DsRed was activated in the presence of ligand and redistributed to a focal pattern in a shorter time period than ERβ1-EGFP in both cell lines. With 10^{-8}M 3βAdiol and the commercial ERβ1-specific agonist DPN™, ERβ1-EGFP took at least 40 minutes to redistribute. However this was only demonstrated over a 60 minute time period with one cell and as observed with the intensity profiles there are differences between cells. The oestrogen receptors ability to redistribute varied within the same experiment between different cells and different cell lines.

Co-transfection studies were performed as it has been proposed that hERα and hERβ can form heterodimers upon ligand binding (Cowley *et al.*, 1997) and this has also been demonstrated using *in vitro* gel shift assays (Moore *et al.*, 1998). To date only one co-localisation study has been published using FP-tagged human ERα and ERβ1 (Matsuda *et al.*, 2002). In the current studies human ERα-DsRed and ERβ1-EGFP did appear to co-localise in the same bright foci in both Hek 293 and Hep G2 cells, consistent with the recent publication using rat ERα and ERβ (Matsuda *et al.*, 2002).

ERα-DsRed and ERβ2-EGFP co-transfected in Hek 293 cells did not appear to co-localise in foci in the presence of E_2 although they were present in the same nucleus, however, intensity profiles showed peaks consistent with both ERs. In Hep G2 cells the intensity profiles suggest that ERα-DsRed and ERβ2-EGFP foci were co-localised and therefore suggest the receptors may have formed heterodimers. As discrete foci containing ERβ2-EGFP were visible only when ERα-DsRed was present it could be postulated that upon heterodimerising with ligand-activated ERα, ERβ2 is "pulled" into the foci together with ERα. This is marked contrast to the behaviour of single transfection studies where ERβ2-EGFP remained in a diffuse distribution within the nucleus even after exposure to E_2.

When ERβ1-DsRed and ERβ2-EGFP were co-transfected the ERs formed a focal, yellow pattern which was more marked in Hep G2 cells compared with Hek 293 cells. The intensity profiles of the ERβ1-DsRed and ERβ2-EGFP suggest that

heterodimers were formed in both cell lines, although more experiments are required to confirm this.

Therefore, for the first time, it has been demonstrated in live cells that the truncated ERβ2 variant protein may form heterodimers with either ERα or ERβ1 in the presence of E_2 (ERα/ERβ2, or ERβ1/ERβ2), although this does not exclude the possibility that homodimers also form in the same nucleus.

In agreement with these results, Matsuda *et al* showed that rat FP-ERα and FP-ERβ1 co-localised in clusters within the nucleus in the presence and absence of ligand (Matsuda *et al.*, 2002). This co-localisation of FP-ERα and FP-ERβ1 suggests that FP-ERα/FP-ERβ1 heterodimers were formed rather than homodimers of each subtype. This is in agreement with biochemical studies which have shown that heterodimers are preferentially formed over each type of homodimer (Pace *et al.*, 1997; Pettersson *et al.*, 1997).

One problem encountered in the present study was the level of transfection efficiency. On average 30% of the cells transfected showed FP-ER clearly visible in the nucleus, some variation in the fluorescent intensity of individual nuclei was also observed. This is in agreement with findings of Htun *et al* who reported a difference in the level of ERα-GFP expression and the overall brightness in different cells and attributed it to cell-to-cell variation (Htun *et al.*, 1999). Therefore in my studies a number of cells were analysed in the same dish, as well as on different occasions so that a more definite conclusion could be made.

These variations between the fluorescence observed in individual cells were exaggerated in co-transfection studies as many cells contained just one of the receptors and those that did contain both sometimes had different levels of fluorescence even though the same amount of plasmid DNA was added to the cell monolayer. These differences may be due to a number of factors including transfection efficiency of the individual cells, the phase of the cell's cycle, physical characteristics, local external environment, co-activators and other regulators present in the cell. The stochastic nature of some of the biological regulatory processes may also contribute to unique nuclear appearance (Htun *et al.*, 1999). For example, Htun *et al* examined the distribution of ERα-GFP in various breast cancer cell lines and noticed that there was a small but significant difference in the

intranuclear distribution of the receptor in the different cells even in the absence of ligand (Htun *et al.*, 1999). In MCF-7 cells (ERα-positive), ERα-GFP was localised in a reticular pattern evenly distributed throughout the nucleus, excluding the nucleous. However, in the ERα-positive T47D cells the ERα-GFP had a composite pattern due to the accumulation of the receptor in a reticular pattern and to a low level of concentration at numerous nuclear sites present throughout the nucleus. In the ERα-negative cell lines, MDA-MB-231 and MDA-MB-435A, ERα-GFP was slightly patchy in appearance therefore indicating that the receptor was unevenly distributed. In these cell lines the extent of redistribution of ERα-GFP to form a focal pattern in the presence of ligand was found to also vary (Htun *et al.*, 1999). Differences in endogenous expression levels may explain the lack of oestrogen dependent changes in nuclear matrix association reported by Htun *et al.*

In this study, in the absence of ligand the transfected FP-ERs were in a mainly diffuse distribution which appeared similar in both the Hek 293 and the Hep G2 cells. This similarity may be due to both cell lines having no detectable ERα or ERβ mRNA or protein prior to transfection (Chapter 3). However in the presence of ligand the receptor redistribution observed varied between the cell lines, this was more notable in the co-transfection studies. This data and previously published data therefore demonstrates differences in the FP-ER distribution that occur in different cell lines in the presence and absence of ligand.

Stenoien *et al* reported that it is necessary to avoid over expression in transiently transfected cells as this leads to incorrect, un-reproducible results (Stenoien *et al.*, 2000). However, in the current studies the concentration of ER tagged to the FP constructs transiently transfected into the cells was comparable to the concentration Stenoien *et al* have used (Stenoien *et al.*, 2000).

In this study, the Hep G2 cells transfected with ERα-DsRed incubated with 10^{-8}M genistein in PBS/Hepes showed a reduced nuclear size and migration of the foci to the nuclear membrane after 130 minutes. Stenoien *et al* demonstrated that the depletion of ATP levels has no effect on the intranuclear mobility of the proteins associated with the splicing speckles and nuclei components (Stenoien *et al.*, 2001a). Therefore in these current studies the change in ER distribution observed after extended incubation in PBS may not be due to ATP depletion but due to depletion of other nutrient factors.

In conclusion, from these studies with FP-tagged ERs it was observed that the addition of ligand induced a redistribution of ERα and ERβ1 from a diffuse to a focal pattern and that this occured rapidly (10 minutes in the presence of 10^{-8}M E_2). In single transfections, ERα and ERβ1 formed foci in the presence of oestrogenic ligand but the truncated ERβ2 which lacks a functional AF-2 domain did not.

Co-transfection of ERα and ERβ1 demonstrated that these ERs can form foci together consistent with previous reports that they heterodimerise as well as homodimerise. ERβ2 has also been shown to form a focal pattern when it is co-expressed with ERα or ERβ1, suggesting that after formation of heterodimers the ERα or ERβ1 has the ability to "pull" the ERβ2 into the foci. The idea that ERβ2 might act as a regulator of ERα and ERβ1 is therefore supported by these findings. These investigations were extended to include functional studies to determine the impact of different ligands on the ability of these receptors to activate ERE-reporters and to determine whether the formation of ERα, ERβ1 and ERβ2 heterodimers affected the ability of the ERs to activate gene transcription.

Chapter 5

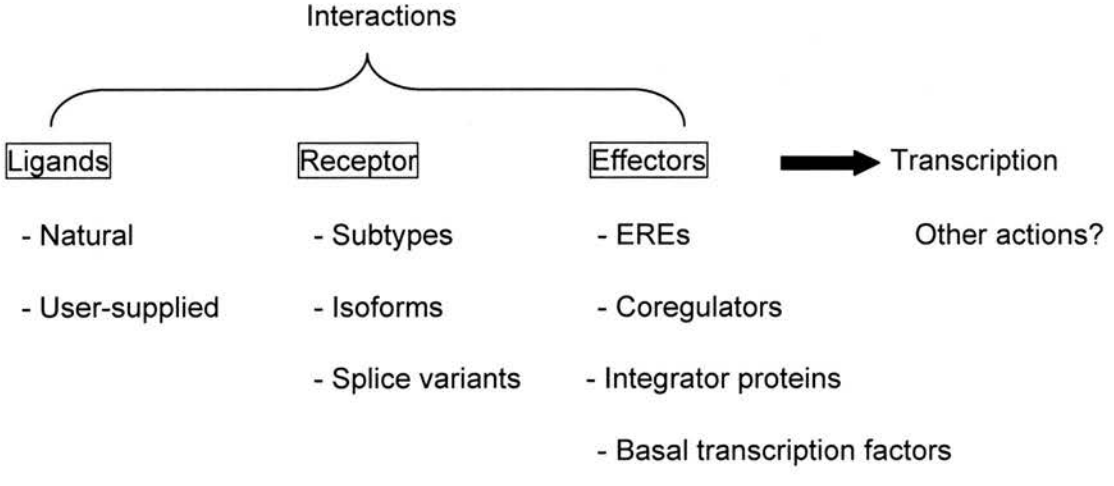
Analysis of the Functional Competence of ER using ERE Reporter Constructs in Cell Transfection Assays

5.1 Introduction

Oestrogens exert profound effects on the physiology of diverse target cells and these effects appear to be mediated by two oestrogen receptors; ERα and ERβ1 (wild type). As observed in Chapter 4, different ligands have the potential to bind to and activate ERα and ERβ1, causing the receptors to redistribute within the nucleus to form a focal or highly concentrated distribution. Functional transient transfections have been performed to ascertain whether this observed redistribution is consistent with an increase in gene transcription.

The pharmacology behind oestrogen action is tripartite (Kraichely *et al.*, 2000). Transcriptional activities of ERα and ERβ1 are influenced not only by ligands, but also by the coregulator proteins with which they associate and the EREs to which they bind to in order to initiate gene transcription.

Figure 5.1. The tripartite nature of nuclear hormone receptor actions involving ligand, receptor and effector interactions. Adapted from Katzenellenbogen *et al.*, (2000).



In this chapter different oestrogen response elements, ligands and co-activators and the effect of co-transfection of ERα and the ERβ variants is examined.

5.1.1 Oestrogen Receptor Interaction with Different Oestrogen Responsive Elements

Stimulation of target gene expression in response to E_2 or other agonists, can be mediated through a "classical pathway" in which the ER binds to a specific oestrogen response element (ERE) on the DNA. This ER-ERE complex then interacts with co-activators and components of RNA polymerase II transcription initiation complex resulting in enhanced transcription (Klinge, 2001). Alternative transcriptional activation pathways, such as the AP-1 mechanism discussed in brief in the literature review, also exist.

It has been documented that differences in ERE sequence impact on ER binding affinity and transcriptional activation (Klinge, 2001). The minimal consensus ERE sequence, present in the *Xenopus laevis* vitellogenin A2 gene (referred to here as Vit ERE), is a 13 bp palindromic inverted repeat; 5'-GGTCAnnnTGACC-3' where "n" is any nucleotide (Wood *et al.*, 2001). It has previously been demonstrated that if the ERE sequence deviates from the consensus sequence by even a single base pair, the receptor exhibits a reduced affinity for the ERE and a decreased transcriptional activity (Wood *et al.*, 2001 and reviewed in the literature review).

Previous studies have shown that ERα and ERβ1 bind to the consensus vitellogenin A2 ERE GGTCAnnnTGACC (Klein-Hitpass *et al.*, 1988) and activate transcription. Human ERβ has a lower affinity for the A2 ERE and a decreased ability to activate transcription of promoters containing the A2 ERE compared with the ERα (Cowley *et al.*, 1997; Kuiper *et al.*, 1996; McInerney *et al.*, 1998b). Although the vitellogenin A2 gene has been extensively used to study oestrogen-regulated gene expression it is important to remember that most endogenous genes contain EREs that differ from the A2 ERE by one or more base pairs and that these differences in nucleotide sequence most likely influences the ability of individual EREs to mediate transcriptional activation. Previous studies have suggested that the ability of individual EREs to modulate receptor conformation may partially account for the differences in transactivation observed with the imperfect EREs (Loven *et al.*, 2001).

In this study reporter constructs containing either the single oxytocin (OT) ERE, the single perfect vitellogenin A2 (Vit) ERE or a three ERE repeat; Vit ERE (3x ERE) were used to assess the different transcriptional activities of ERα and ERβ in response to E_2.

5.1.2 Different Ligands which Activate ERα and ERβ1

It has been shown in Chapter 4 that FP tagged ERα and ERβ1 proteins respond to addition of different ligands in transiently transfected cells by redistributing into a focal nuclear pattern. To follow on from these observations, functional studies were performed to assess whether these changes in ER distribution upon addition of ligand initiated gene transcription via an ERE reporter.

Previous studies have analysed the binding abilities and the transcriptional activation of the ERs with different ligands (Barkhem *et al.*, 1998; Kuiper *et al.*, 1996). As the sequence of the LBD of the ER isofoms is not identical and different conformational changes are initiated upon ligand binding, the activation of gene transcription is proposed to vary.

Some of the ligands used in this chapter have been shown to bind to the LBD of ERα and ERβ1 and cause initiation of gene transcription to different extents (Barkhem *et al.*, 1998; Kuiper *et al.*, 1998). E_2 binds to both ERα and ERβ1 whereas 3βAdiol, a metabolite of DHT, is proposed to work as an antagonist for ERα but an agonist for ERβ1 (Weihua *et al.*, 2003). Genistein is a phytoestrogen which is reported to activate ERβ1 to a greater extent than ERα (Liu *et al.*, 2003; Pettersson and Gustafsson, 2001; Pike *et al.*, 1999). The novel ligand PPT™ is ERα-specific, whereas DPN™ is proposed to be an ERβ1-specific ligand (Kraichely *et al.*, 2000; Meyers *et al.*, 2001).

Dose response studies were performed to assess the concentration required to stimulate ER activation and the concentrations which resulted in maximal transcriptional activation. Human ERα, ERβ1 and ERβ2 as well as ERβ1 cloned from macaque and marmoset were investigated to assess their ability to initiate gene transcription with different ligands using a 3x ERE-Luc reporter. To my knowledge there is no literature published on primate ERβ responses to different ligands.

5.1.3 The Effects of Co-transfection with ERα, ERβ1 and ERβ2 Variant

Immunohistochemical analysis described in Chapter 3 has demonstrated that the ERα and ERβ1 isoforms and the ERβ2 splice variant can be expressed within the same tissues. If the receptors are expressed together in the same nucleus they have the ability to form heterodimers and under experimental conditions these appear to form in preference to homodimers (Cowley *et al.*, 1997). The behaviour of combinations of FP tagged ERα and ERβ following co-transfections in the same cell nucleus has been investigated in Chapter 4. It was observed that when ERβ2 was co-transfected with ERα or ERβ1 it was able to redistribute into a focal pattern together with either ERα or ERβ full length receptor. This observation is consistent with reports that ERβ2 can heterodimerise with ERα or ERβ1 in gel shift assays (Cowley *et al.*, 1997; Moore *et al.*, 1998).

ERα and ERβ1 have been shown not to be functionally equivalent (Hall and McDonnell, 1999; Pettersson *et al.*, 1997). For example in many contexts ERα is significantly more transcriptionally active than ERβ1 and it has been proposed that ERβ1 could act as a modulator of ERα transcriptional activity (Cowley *et al.*, 1997; Hall and McDonnell, 1999; Weihua *et al.*, 2000). Previously, co-transfection studies have been carried out to analyse the effects of ERβ1 on the transcriptional activity of ERα (Cowley *et al.*, 1997; Ogawa *et al.*, 1998a; Pettersson *et al.*, 2000). However, more recently, the identification of the ERβ2 variant lacking part of the LBD including the AF-2 region (Moore *et al.*, 1998; Ogawa *et al.*, 1998c) has led to suggestions that ERβ2 may act as a dominant negative regulator of ERα activity (Ogawa *et al.*, 1998c). Inoue *et al* co-transfected an ERβ isoform lacking all of the LBD (Δ5) with ERα or ERβ1 wild type and reported a slight dominant negative effect on ERα, but an increase in ERβ1's activity (Inoue *et al.*, 2000). As the ERβ isoforms are co-expressed in cells in many tissues, including the testis and endometrium (Chapter 3 and Saunders *et al.*, 2000; Saunders *et al.*, 2002a; Saunders, 2003) it is important to investigate the functional competence of the ERβ isoforms. Therefore in this chapter functional studies were performed to assess transcriptional activity of ERα, ERβ1 and the ERβ2 variant as well as the effect of co-expression of these receptors in the same cells.

5.1.4 Steroid Receptor Co-activators (SRC)

A number of previous studies have identified co-activator and co-repressor proteins capable of interacting with nuclear receptors and influencing their transcriptional activity (McKenna and O'Malley, 2002; Onate *et al.*, 1995). The steroid receptor co-activator (SRC) was the first co-activator to be identified (Onate *et al.*, 1995) and has been found to influence ERα or ERβ gene transcription (Gee *et al.*, 1999; Wong *et al.*, 2001) and to be affected by different EREs (Hall *et al.*, 2002).

Three members of the SRC gene family have been identified in human and rodents; SRC-1, -2, and -3 (Xu and O'Malley, 2002). All three members of the SRC family are able to interact with ERs in a ligand dependent manner and significantly enhance ER transcriptional activity (Xu and O'Malley, 2002). The SRC-1 isoform acts to initiate reporter gene transcription by binding to ERα and ERβ1 following ligand binding and dimerisation. In addition to ERs, SRC co-activators can also interact with and activate other members of the steroid hormone receptor superfamily including androgen and progesterone receptors (Lee *et al.*, 1998; Leo and Chen, 2000). Human proteins encoded by the SRC gene family are approximately 160 kDa in size and have an overall sequence similarity of 50 to 55% and sequence identity of 43 to 48%. Their most conserved region is the N-terminal domain which is involved in DNA binding and heterodimerisation between proteins containing similar motifs.

Two functionally distinct SRC-1 isoforms have been identified, SRC-1e and SRC-1a, which contain unique C-termini therefore suggesting that alternative splicing may regulate SRC function (Kalkhoven *et al.*, 1998). The two human SRC isoforms are identical up to residue 1385 but differ at their C-termini. SRC-1a (1441 amino acids; 156.7 kDa) contains 56 unique residues and lacks 14 of the most C terminal amino acids present in SRC-1e (1399 amino acids; 152.3 kDa) (Kalkhoven *et al.*, 1998). Both isoforms contain three nuclear receptor binding motifs (LXXLL) and an activation domain which co-localises with the CBP-binding domain, whilst SRC-1a contains a unique LXXLL motif in its C-terminus. The relative expression of the two SRC isoforms has been studied by RNase protection assays (Kalkhoven *et al.*, 1998) and both were detected in all the cell lines tested, including the Hek 293 and Hep G2 cells used in the current study.

5.1.5 Aims

The aims of this chapter were to examine the functional differences between different EREs and to examine the dose responses of ER proteins with different ligands using transient transfections, in the presence of an ERE-reporter construct. The effect of co-transfecting the different ER isoforms and the co-transfection of SRC-1 with the ERs, to alter gene transcription was also assessed.

5.2 Methods

Transient transfections were carried out as in section 2.10 using the Hek 293 or the Hep G2 cell lines.

5.2.1 Plasmids

All the plasmids used in transient transfection studies were purified using CsCl preparations described in section 2.9.2.3.

5.2.1.1 Oestrogen Receptors in pcDNA3.1 Vector

Human, macaque and marmoset full length ERβ1 and ERβ2 cDNAs and human ERα cDNA were cloned into the pcDNA3.1 (Invitrogen) vector (section 2.7.3.5).

5.2.1.2 Oestrogen Receptor Reporter Constructs

The EREs used were tagged to the luciferase promoter construct so their response to ER activation in transient transfections could be analysed using the Luciferase assay (section 2.10.2.4). The sequences of the EREs are detailed in *Table 5.1*.

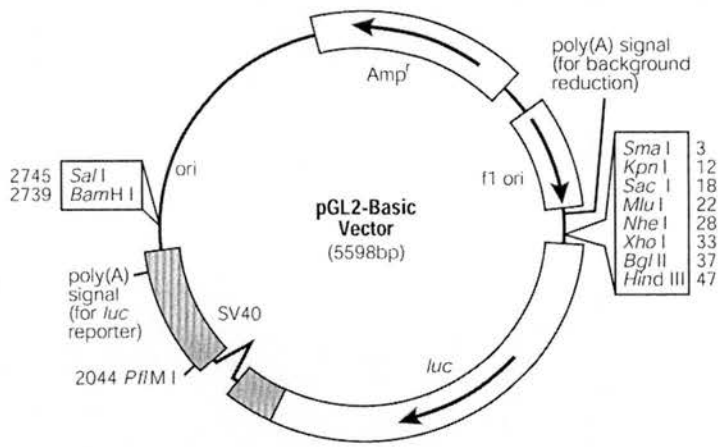
Table 5.1 Details of the different ERE sequcences.

ERE	ERE Sequence
3x ERE	tAGGTCAcagTGACCtgcggatccgcAGGTCActgTGACCtagatccgcAGGTC ActgTGACCt
OT ERE	GGTGAcctTGACC
pTAL ERE	AGGTCAcagTGACCt
Vit ERE (PGL)	AGGTCAcagTGACCt

The significant differences in sequence are underlined.

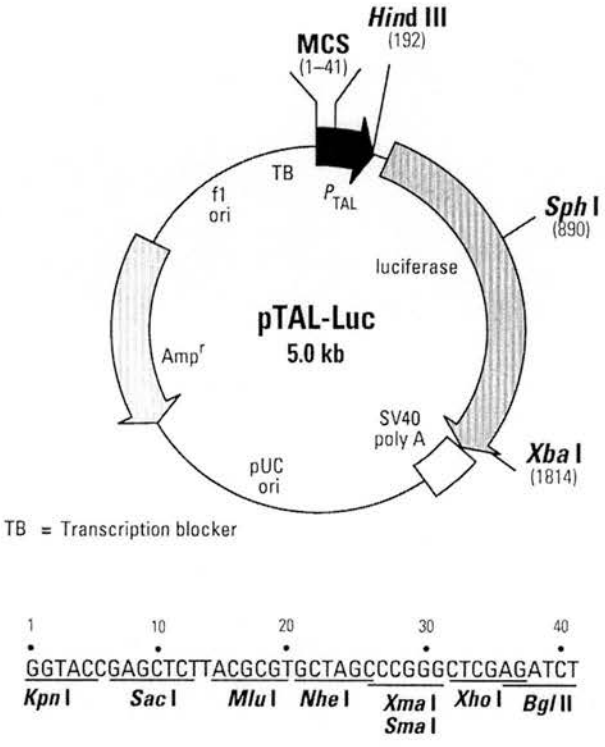
The bovine oxytocin (OT) ERE, mutated to resemble rat ERE, was obtained from Norbert Walther (Institute for Hormone and Fertility Research, University of Hamburg, Germany. (Adan *et al.*, 1991)). The OT (approximately 160 bp) was already tagged to the luciferase promoter and had been cloned into pGL2 Basic Vector (Promega) between SmaI and HindIII restriction sites (*Figure 5.2*).

Figure 5.2. Diagram with details of the pGL2-basic vector used for the OT ERE and Vit ERE.



The A2 vitellogenin ERE sequence was cloned into pGL2-Basic-Luciferase vector (Promega) (*Figure 5.2*) and the pTAL-Luciferase vector (Promega) (*Figure 5.3*) to form Vit ERE (pGL) and pTAL ERE respectively (Table 5.1). The work was performed by Graeme Scobie (MRC Human Reproductive Sciences Unit).

Figure 5.3. Diagram with details of the pTAL-basic vector used for the Vit ERE.



The 3x ERE-TK-Luc reporter construct was a gift from S.C. Nagel and D.P. McDonnell (Department of Pharmacology and Cancer Biology, Duke University Medical Centre, North Carolina, USA. (Nagel *et al.*, 2001)). It contained three tandem copies of the vitellogenin ERE that had been ligated into the TK-Luciferase vector (Ligand Pharmaceuticals) using Bgl II and Hind III.

5.2.1.3 Steroid Receptor Co-activator-1 (SRC-1)

SRC-1e and SRC-1a constructs were a kind gift from Professor M. Parker (Molecular Endocrinology Laboratory, Imperial Cancer Research Fund, London, UK. (Kalkhoven *et al.*, 1998)) and had been inserted into the pSG5 vector. The SRC-1 constructs were supplied on blotting paper so the spots were reconstituted in TE, transformed in XLI Blue cells (Stratagene) and plated onto amplillicin-containing agar plates (section 2.9.1). Prior to use in transient transfection studies the plasmids were purified using the CsCl technique (section 2.9.2.3).

5.2.1.4 Renilla Internal Control

The pRL-CMV vector (Promega) contains the CMV enhancer and early promoter elements which provide a high level expression of Renilla luciferase. When co-

transfected with the ERE-luciferase constructs this provides an internal control for the Luciferase assay. The pRL-CMV construct was transformed in XLI Blue cells (Stratagene), plated out and propagated in LB (section 2.9.1). A CsCl preparation (section 2.9.2.3) was performed so that the constructs were prepared for transfection.

5.2.2 Luciferase Assay

The Dual-Luciferase® Reporter Assay (Promega) was used to measure the level of transcriptional activity as described in section 2.10.2. The experimental ERE-Luc reporter activity was corrected against the Renilla internal control. This normalisation of the luciferase activity was essential as it minimises the experimental variability caused by the differences between cell viability or transfection efficiency. The results were expressed as a fold increase in activity over the control, ie. the luciferase value given for constructs transfected into the cells in the absence of ligand.

5.2.3 Transient Transfection Studies

The transfections were carried out as described in section 2.10.2. For every experiment, each condition was performed in duplicate and the experiment performed a minimum of three times in a 12 well plate.

JetPEI transfection reagent (Cambridge Biosciences) was used for transfecting the cells (section 2.10.2.2) in all of the experiments except for the Hek 293 E_2 dose response and the Hek 293 co-transfection studies. In these experiments Gene Porter (Gene Therapy Systems) was used (section 2.10.2.1).

5.2.3.1 Activation of ERE-Luciferase Reporters

For a comparison of different ERE-Luc reporters in Hek 293 and Hep G2 cells, 1μg of the ER, 0.1μg pRL-CMV and 0.25μg of the ERE-Luc was used in each well in a 12 well plate. To each either ethanol vehicle, 10^{-8}M or 10^{-7}M E_2 was added 4 hours after transfections (see *Table 5.2*).

For a comparison of different concentrations of the 3xERE, three conditions were used. Either 0.125μg, 0.25μg or 0.5μg 3xERE were added to 0.1μg pRL-CMV only, or to 1μg ER and 0.1μg pRL-CMV in each well. Vehicle or 10^{-8}M E_2 was then added to the transfected cells 4 hours later (see *Table 5.2*).

Table 5.2. Details of the different EREs used and their concentrations.

	ER	ERE	pRL-CMV	E_2 Conc.
Different EREs	1μg	0.25μg 3xERE, OT ERE, pTAL ERE or Vit ERE	0.1μg	Vehicle, 10^{-8}M & 10^{-7}M
3xERE Concentrations	1μg	0.125μg, 0.25μg & 0.5μg	0.1μg	Vehicle & 10^{-8}M

5.2.3.2 Dose Response Studies

Using the Hek 293 and the Hep G2 cells, 1μg ER, 0.5μg 3x ERE and 0.2μg pRL-CMV was used in each well of the 12 well plate. Different ligands were added to the cells four hours after transfection. E_2 and 3βAdiol ligands were added to Hek 293 cells; E_2, 3βAdiol, genistein, PPT™ and DPN™ were added to Hep G2 cells, with concentrations ranging from 10^{-14}M to $2.5x10^{-5}$M.

5.2.3.3 Co-transfection Studies

Using Hek 293 and Hep G2 cells, 200ng of the first ER was added, with either 200ng (1:1 ratio), 600ng (1:3) or 1200ng (1:6) of the second ER, 0.25μg 3x ERE and 0.1μg pRL-CMV. Four hours after transfection, different concentrations ranging from 10^{-13}M to 10^{-7}M, of E_2 were added to the cells.

5.2.3.4 Impact of SRC-1 in Single ER Transfections or ER Co-transfections

Co-transfections were carried out in Hep G2 cells. In each well, 1μg ER, 0.25μg 3x ERE and 0.1μg pRL-CMV were added, with 1μg of the SRC-1e or SRC-1a constructs. Four hours after transfection, either an ethanol vehicle, 10^{-8}M or 10^{-7}M E_2 was added to the wells.

In co-transfection studies using two ERs and SRC-1e in Hep G2 cells; 200ng of the first ER was transfected with either 200ng, 600ng or 1200ng of the second ER along with 1μg SRC-1e, 0.25μg 3xERE and 0.1μg pRL-CMV. Either an ethanol vehicle or 10^{-8}M E_2 was added four hours after transfection.

5.3 Results

Each luciferase value was normalised to the internal pRL-CMV Renilla internal control value and fold activation was calculated by dividing the values by the vehicle control (set at 1). Each data point is the average of duplicate experiments performed on at least three occasions. The standard error is shown for all data.

5.3.1 Activation of ERE-Luciferase Reporter

5.3.1.1 Comparison of the Different ERE Reporter Constructs

Hek 293 cells (*Figure 5.1*) or Hep G2 cells (*Figure 5.2*) were co-transfected with the luciferase reporter plasmids containing the different ERE sequences. The results were normalised and expressed as a fold increase compared to the ERE-Luc alone (*Figure 5.1A*) or ERE-Luc and hER without E_2 (*Figures 5.1B & 5.2*).

In the presence of E_2 the fold induction of the luciferase reporter was influenced by the promoter sequence and by whether human ERα or hERβ1 was introduced into the cells. Overall, the 3x ERE-containing reporter construct showed the greatest fold increase compared to the single EREs and the OT ERE (*Table 5.3*) in both Hek 293 and Hep G2 cells.

In Hek 293 cells (*Figure 5.1A*) the OT ERE co-transfected with hERα induced a high level of transcriptional activity, resulting in increased activity greater than that of the 3x ERE, notably at 10^{-7}M ligand. A similar level of induction was not observed with hERβ1 in the Hek 293 cells or by hERα and hERβ1 in the Hep G2 cells. In Hek 293 and Hep G2 cells transfected with hERα, the pTAL ERE and Vit ERE (pGL2 reporter) showed a similar, minimal increase in transcriptional activity in the presence of 10^{-7}M E_2.

Therefore the 3x ERE-Luc reporter construct was used in all subsequent studies as it initiated the greatest transcriptional response with hERα and hERβ1 in the presence of E_2 in both cell lines as shown in *Table 5.3*.

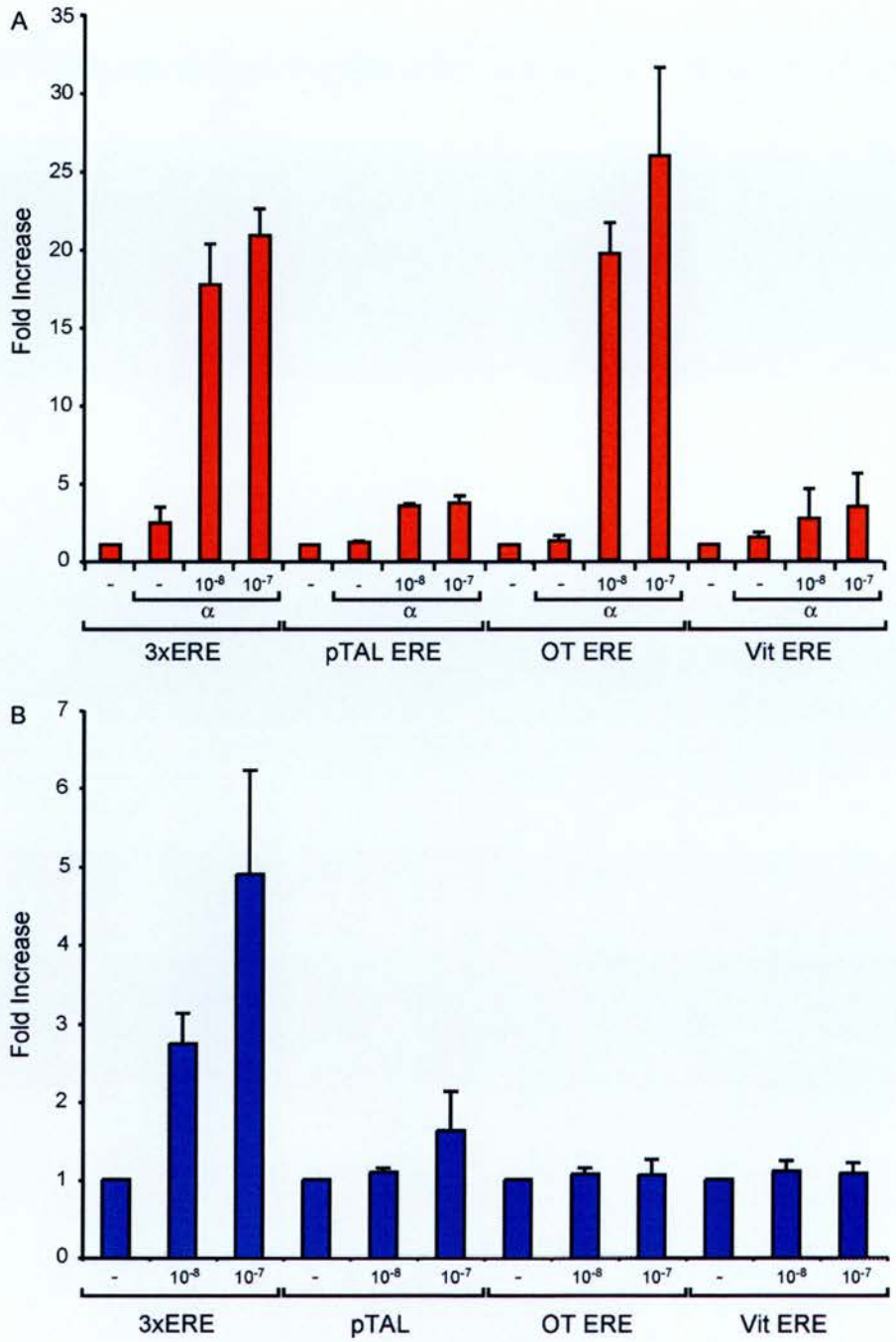


Figure 5.1. Comparison of the different EREs and their ability to activate gene transcription in Hek 293 cells. Hek 293 cells were co-transfected with the luciferase reporter plasmid containing the 3x ERE, pTAL, OT or Vit ERE and the pRL-CMV internal control, ERα (A) or ERβ1 (B) were added. The cells were treated with vehicle, 10^{-8}M or 10^{-7}M E_2. Each value was normalised to the internal pRL-CMV Renilla control and fold activation was calculated by dividing values by the vehicle control (set at 1). NB in (A) the control is the ERE alone, in the absence of ERα, (B) control includes ERβ1. Each data point is the average of duplicate determinations from three representative experiments. Scales presented are different. Standard errors are calculated from the means of the three experiments.

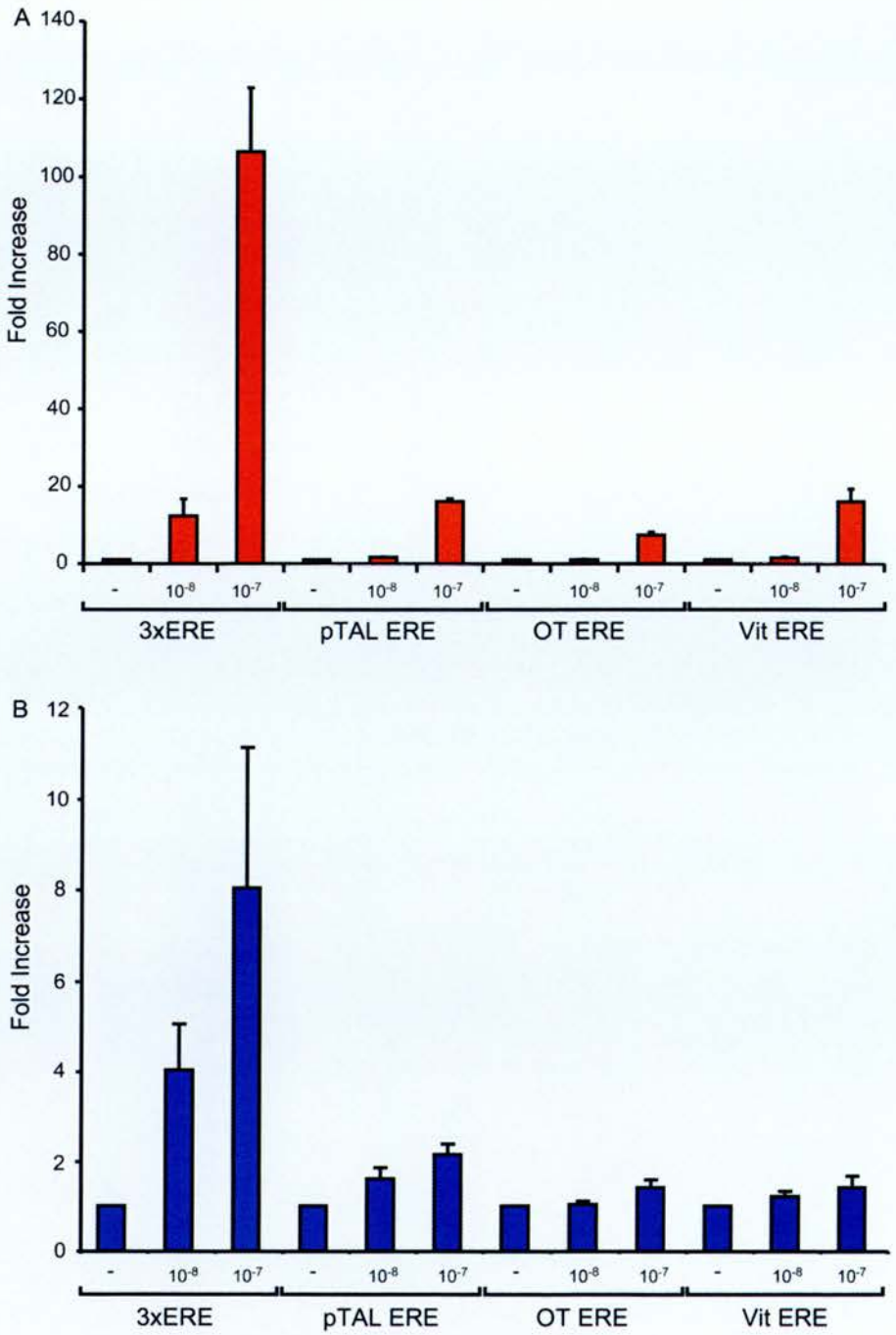


Figure 5.2. Comparison of the different EREs and their ability to activate gene transcription in Hep G2 cells. Hep G2 cells were co-transfected with the luciferase reporter plasmid containing the 3x ERE, pTAL, OT or Vit ERE and the pRL-CMV internal control, ERα (A) or ERβ1 (B) were added. The cells were treated with vehicle, 10^{-8}M or 10^{-7}M E_2. Data are displayed as fold induction of luciferase activity over the pRL-CMV internal control, scales presented are different. Data are the mean +/- SEM of 3 experiments.

Table 5.3. Summary of the effect of different promoter sequences on induction of the reporter expression in the presence of E_2. Values given as the mean fold increase over the control. This being the selected ERE in the absence of ligand but with addition of the appropriate constructs.

Cell line	ER	3x ERE		pTAL		OT ERE		Vit ERE	
		10^{-8}M	10^{-7}M	10^{-8} M	10^{-7} M	10^{-8} M	10^{-7} M	10^{-8}M	10^{-7}M
Hek 293	ERα	17.7	20.8	3.5	3.7	19.7	26.0	2.7	3.5
Hek 293	ERβ1	2.7	2.9	1.1	1.6	1.1	1.1	1.2	1.1
Hep G2	ERα	12.4	106.3	1.7	16.1	1.0	7.5	1.5	16.0
Hep G2	ERβ1	4.0	8.1	1.6	2.1	1.0	1.4	1.2	1.4

5.3.1.2 Comparing Different Concentrations of 3xERE with ERα and ERβ1

Increasing concentrations of the 3x ERE-Luc reporter construct co-transfected with a fixed amount of human ERα or hERβ1 in Hep G2 cells was assessed. The higher the concentration of the 3x ERE-Luc reporter construct used for transient transfections, the greater the transcriptional activity detected by the luciferease assay and the higher the observed fold increase (*Figure 5.3* & *Table 5.4*).

Table 5.4. Summary of the effect of different concentrations of the 3x ERE-Luc reporter construct on gene transcription with 10^{-7}M E_2 in Hep G2 cells. Values given as a mean fold increase over the normalised condition in the absence of ligand.

3x ERE	0.125μg	0.25μg	0.5μg
ERα	12.0	35.3	47.0
ERβ1	1.8	5.0	10.0

Therefore, in the subsequent studies the ER constructs were co-transfected with 0.5μg of the 3x ERE-Luc reporter construct.

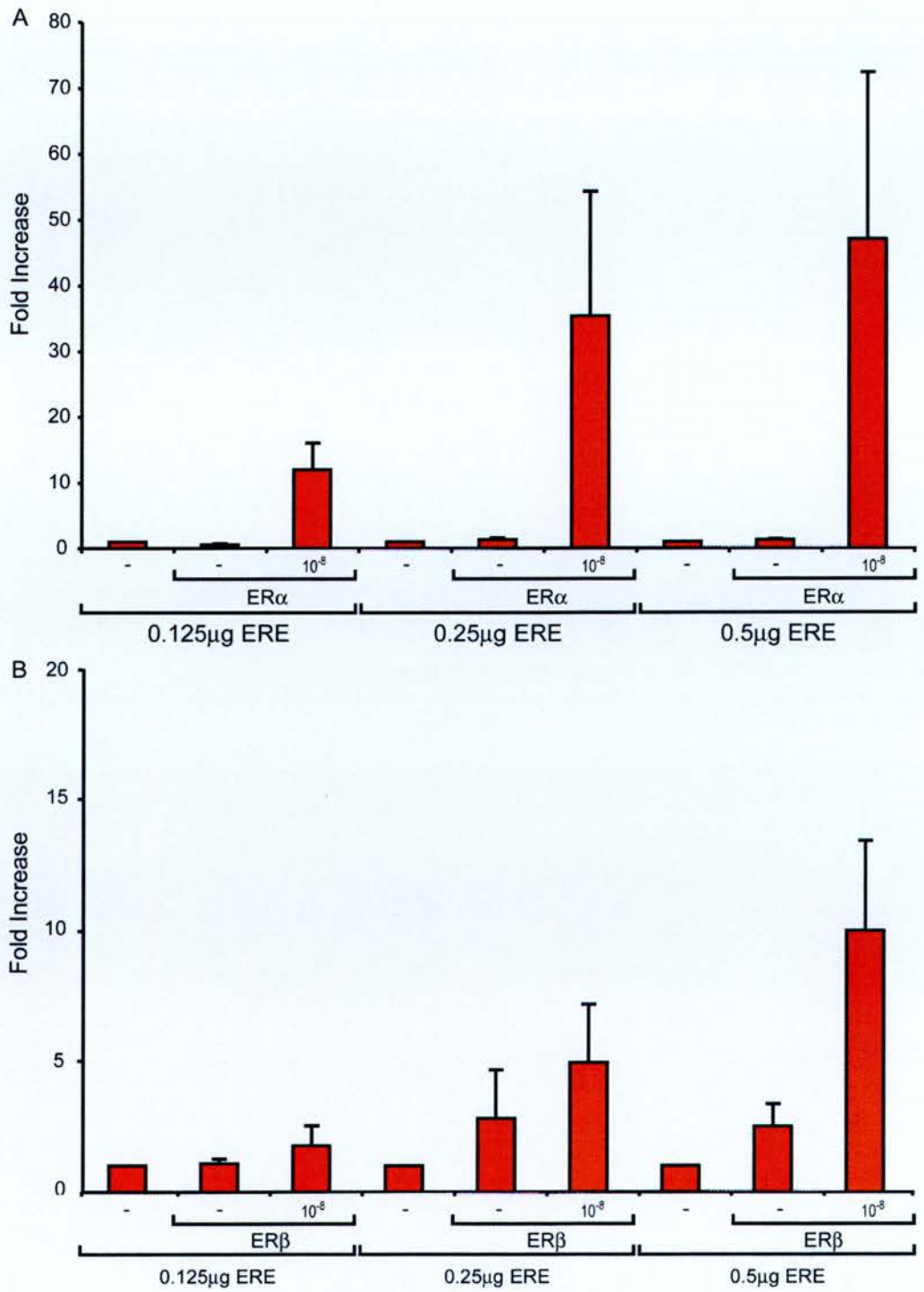


Figure 5.3. Assessing the impact of different amounts of 3x ERE-Luc co-transfected with hERα or hERβ. Hep G2 cells were co-transfected with the hERα (A) or hERβ1 (B), the pRL-CMV internal control and either 0.125µg, 0.25µg or 0.5µg 3x ERE-luciferase reporter plasmid. The cells were treated with vehicle or 10^{-8}M E_2. Data are displayed as fold induction of luciferase activity over the pRL-CMV internal control, scales presented are different. Data are the mean +/- SEM of 3 experiments.

5.3.2 Impact of Steroid Hormones on Transcriptional Activity of ERs

5.3.2.1 17β-Oestradiol Dose Response

In Hek 293 and Hep G2 cells the 3x ERE-Luc reporter construct transiently transfected together with human ERα or human, macaque or marmoset ERβ1, showed a dose response curve in activity in the presence of increasing concentrations of E_2 (10^{-14}M to 10^{-6}M) (*Figures 5.4* & *5.5*).

The different ER constructs in the Hek 293 and the Hep G2 cells showed similar ERE reporter gene activation potential. The hERα construct activated the greatest increase in activity of the 3x ERE-Luc reporter gene compared to the ERβ1 constructs and required a lower concentration of E_2 to activate maximal activity (Hek 293 cells). However the E_2 concentration required to give maximum activation of the 3x ERE-Luc reporter varied. For example, maximal activation was achieved when 10^{-7}M E_2 was added to hERα in Hek 293 cells, but was reached when 10^{-6}M E_2 was added to ERα transfected into Hep G2 cells.

The hERβ2-containing construct failed to induce transcription of the 3x ERE-Luc reporter gene in the presence of E_2 (from 10^{-14}M to 10^{-6}M) in the Hek 293 or the Hep G2 cells.

Transcriptional activity of macaque and marmoset ERβ1 (*Figures 5.4D, E* & *5.5.D, E*) was similar to hERβ1 in both cells lines (see *Tables 5.4* & *5.5*).

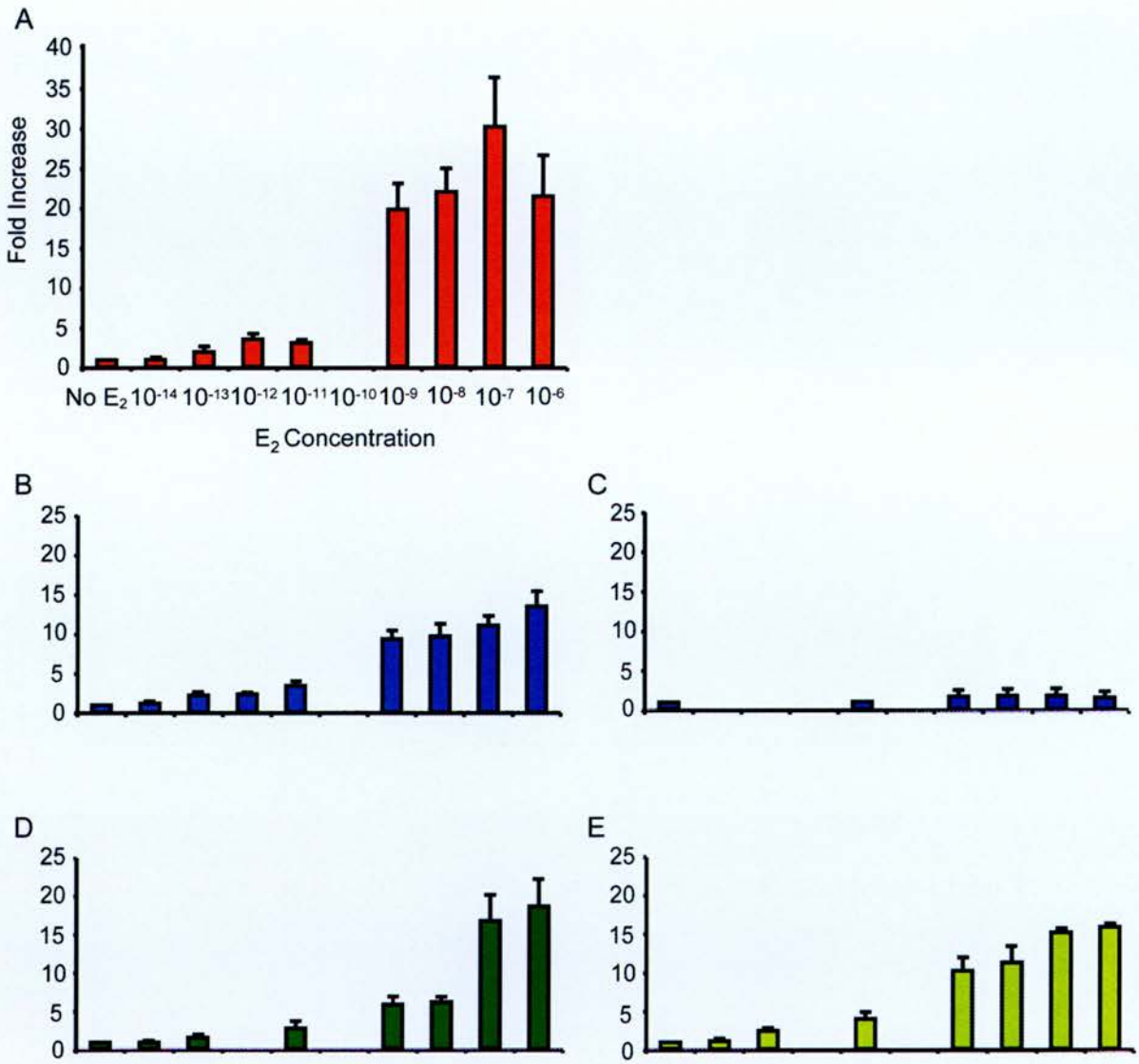


Figure 5.4. 17β-Oestradiol dose response with ER transfected Hek 293 cells. Hek 293 cells were transiently transfected using Gene Porter transfection reagent, with hERα (A), hERβ1 (B), hERβ2 (C), Mac ERβ1 (D) or Marm ERβ1 (E), the 3x ERE-Luc reporter and the pRL-CMV internal control. Cells were treated with an ethanol vehicle or increasing concentrations of 17β-oestradiol (E_2), ranging from 10^{-14}M to 10^{-6}M. Data are displayed as fold induction of luciferase activity over the un-stimulated cell. Data are the mean +/- SEM of 3 experiments.

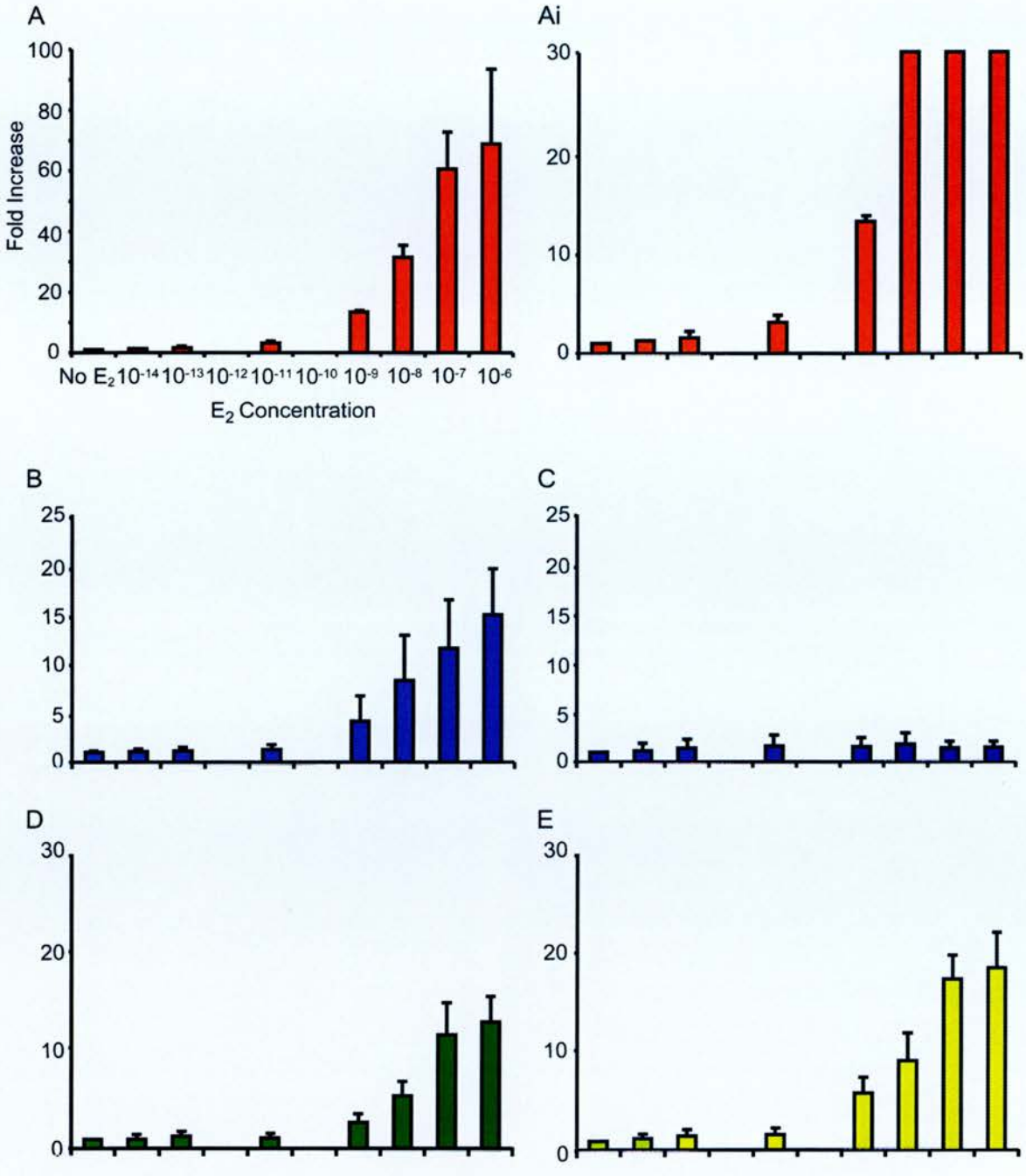


Figure 5.5. 17β-Oestradiol dose response with ER transfected Hep G2 cells. Hep G2 cells were transiently transfected using the JetPEI transfection reagent with hERα (A), hERα; reduced scale (Ai), hERβ1 (B), hERβ2 (C), Mac ERβ1 (D) or Marm ERβ1 (E), the 3x ERE-Luc reporter and the pRL-CMV internal control. Cells were treated with an ethanol vehicle or increasing concentrations of 17β-oestradiol (E_2) ranging from 10^{-14}M to 10^{-6}M. Data are displayed as fold induction of luciferase activity over the un-stimulated cell. Data are the mean +/- SEM of 3 experiments.

5.3.2.2 3βAdiol Dose Response

In both Hek 293 and Hep G2 cells 3βAdiol induced transcription of the 3x ERE reporter gene by hERα and human, macaque and marmoset ERβ1 in a dose dependent manner (*Figures 5.6* & *5.7*).

In the Hek 293 cells transfected with hERα, transcriptional activity of the ERE reporter increased gradually to reach a maximum with 10^{-7}M 3βAdiol. In the Hep G2 cells transfected with hERα, low concentrations of 3βAdiol (10^{-14}M to 10^{-8}M) induced minimal reporter gene activation. However the addition of 10^{-7}M and 10^{-6}M 3βAdiol induced a large increase in activation of the reporter gene (67.6-fold and 415.8-fold respectively) with ERα. This large increase in the reporter gene activation was not observed with any of the ERβ1-containing constructs in either cell line. This high induction of transcriptional activity may be due to a downward trend of Renilla expression at high 3βAdiol concentrations. At lower concentrations (10^{-14}M to 10^{-8}M) human, macaque and marmoset ERβ1 initiated ERE reporter gene transcription greater than hERα (see *Tables 5.4* & *5.5*) in both cell lines. The marmoset and especially the macaque ERβ1 initiated a greater increase in ERE reporter gene activation compared to hERβ1 in Hep G2 cells. The macaque ERβ1 was observed to increase the ERE activity to a greater extent at lower concentrations (10^{-14}M to 10^{-7}M) of 3βAdiol than the marmoset ERβ1 in both cell lines.

The hERβ2-containing construct failed to induce transcription of the 3x ERE-Luc reporter gene in the presence of 3βAdiol (10^{-14}M to 10^{-6}M) in either the Hek 293 or the Hep G2 cells.

5.3.2.3 Genistein Dose Response

Genistein induced a dose responsive induction of the 3x ERE reporter gene transcription when hERα or ERβ1 were transiently transfected into Hep G2 cells (*Figure 5.8*). Transfection of hERα initiated the lowest fold induction compared to the ERβ1 constructs with all doses of genistein (10^{-14}M to 10^{-7}M). The human and marmoset ERβ1 initiated the greatest level of ERE reporter gene activation at high (10^{-7}M to 10^{-6}M) doses of genistein (see *Table 5.5*). The human ERβ2 construct did not induce reporter gene activity when it was expressed in Hep G2 cells and exposed to increasing concentrations of genistein (10^{-14}M to 10^{-6}M).

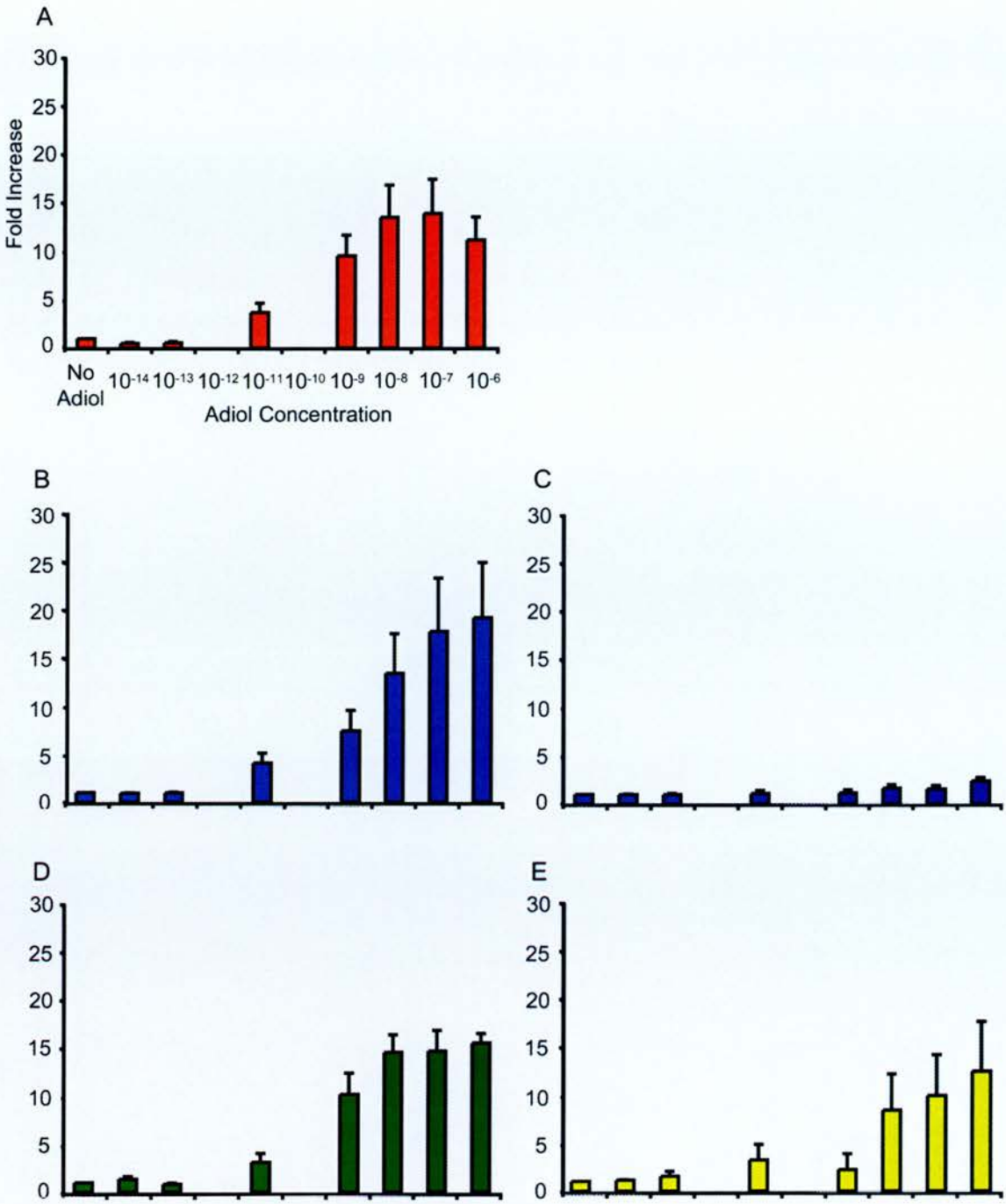


Figure 5.6. 3βAdiol dose response in Hek 293 cells. Hek 293 cells were transiently transfected with hERα (A), hERβ1 (B), hERβ2 (C), Mac ERβ1 (D) or Marm ERβ1 (E), the 3x ERE-Luc reporter and the pRL-CMV internal control. Cells were treated with a vehicle, or increasing concentrations of 3βAdiol, ranging from 10^{-14}M to 10^{-6}M. Data are displayed as fold induction of luciferase activity over the un-stimulated cell. Data are the mean +/- SEM of 3 experiments.

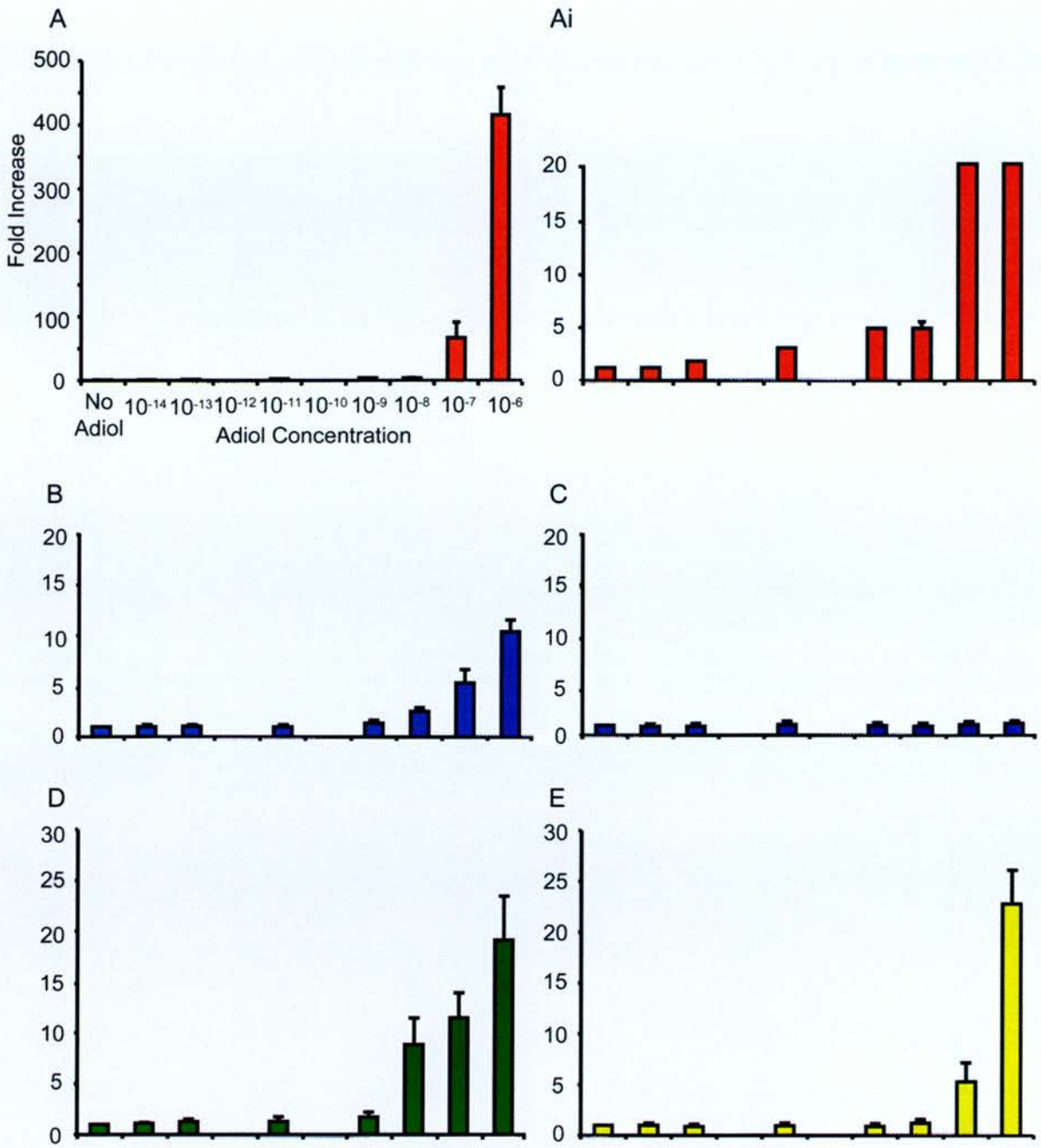


Figure 5.7. 3βAdiol dose response in Hep G2 cells. Hep G2 cells were transiently transfected with hERα (A), hERα; reduced scale (Ai), hERβ1 (B), hERβ2 (C), Mac ERβ1 (D) or Marm ERβ1 (E), the 3x ERE-Luc reporter and the pRL-CMV internal control. Cells were treated with a vehicle, or increasing concentrations of 3βAdiol, ranging from 10^{-14}M to 10^{-6}M. Data are displayed as fold induction of luciferase activity over the un-stimulated cell. Data are the mean +/- SEM of 3 experiments.

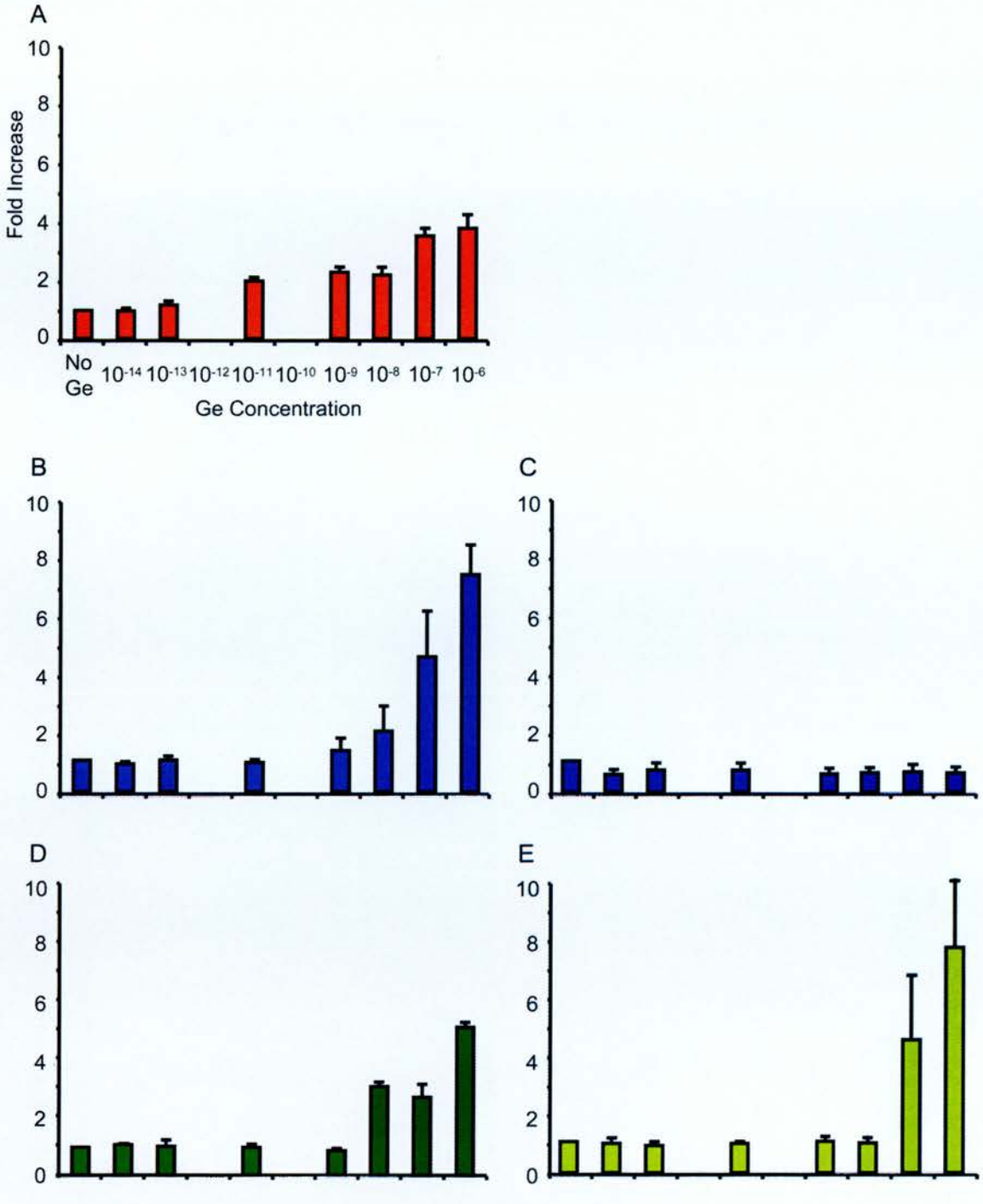


Figure 5.8. Genistein dose response in Hep G2 cells. Hep G2 cells were transiently transfected with hERα (A), hERβ1 (B), hERβ2 (C), Mac ERβ1 (D) or Marm ERβ1 (E), the 3x ERE-Luc reporter and the pRL-CMV internal control. Cells were treated with a vehicle, or increasing concentrations of genistein (Ge), ranging from 10^{-14}M to 10^{-6}M. Data are displayed as fold induction of luciferase activity over the un-stimulated cell. Data are the mean +/- SEM of 3 experiments.

5.3.2.4 PPT™ Dose Response

The ERα-specific agonist PPT™ induced reporter gene transcription in the presence of human ERα, macaque or marmoset ERβ1 using Hep G2 cells (*Figure 5.9*). Transcriptional activity of the 3x ERE reporter was upregulated in a dose dependent manner. With hERα, transcriptional activity of the reporter became apparent when 10^{-9}M PPT™ was present and the greatest fold increase in activity was demonstrated with 10^{-6}M PPT™. Human ERβ1 and ERβ2 did not initiate gene transcription in the Hep G2 cells with any concentration of PPT™. However, macaque ERβ1 induced reporter gene activation with PPT™ at 10^{-8}M and marmoset stimulated reporter activity slightly with 10^{-6}M PPT™ (see *Table 5.5*).

5.3.2.5 DPN™ Dose Response

The ERβ1-specific agonist DPN™ activated hERα and ERβ1 in Hep G2 cells (*Figure 5.10*). Human ERβ1 showed an increase in ERE-Luc reporter gene activity when the cells were treated with 10^{-9}M DPN™. When 10^{-8}M DPN™ was added to the cells transfected with hERβ1, the 3xERE reporter gene activation reached a maximum and remained at a constant level of transcriptional activation. Human ERα induced gene transcription when DPN™ was added to the cells at concentrations of 10^{-6}M and higher and initiated a greater level of reporter gene activity compared to ERβ1 with concentrations of 10^{-6}M and above (see *Table 5.5*).

The marmoset and macaque ERβ1-containing constructs stimulated gene transcription in a dose dependent manner in the presence of DPN™, with marmoset ERβ1 showing the greatest increase in reporter gene activation.

None of the concentrations of DPN™ tested were able to induce reporter gene activation by the hERβ2 construct.

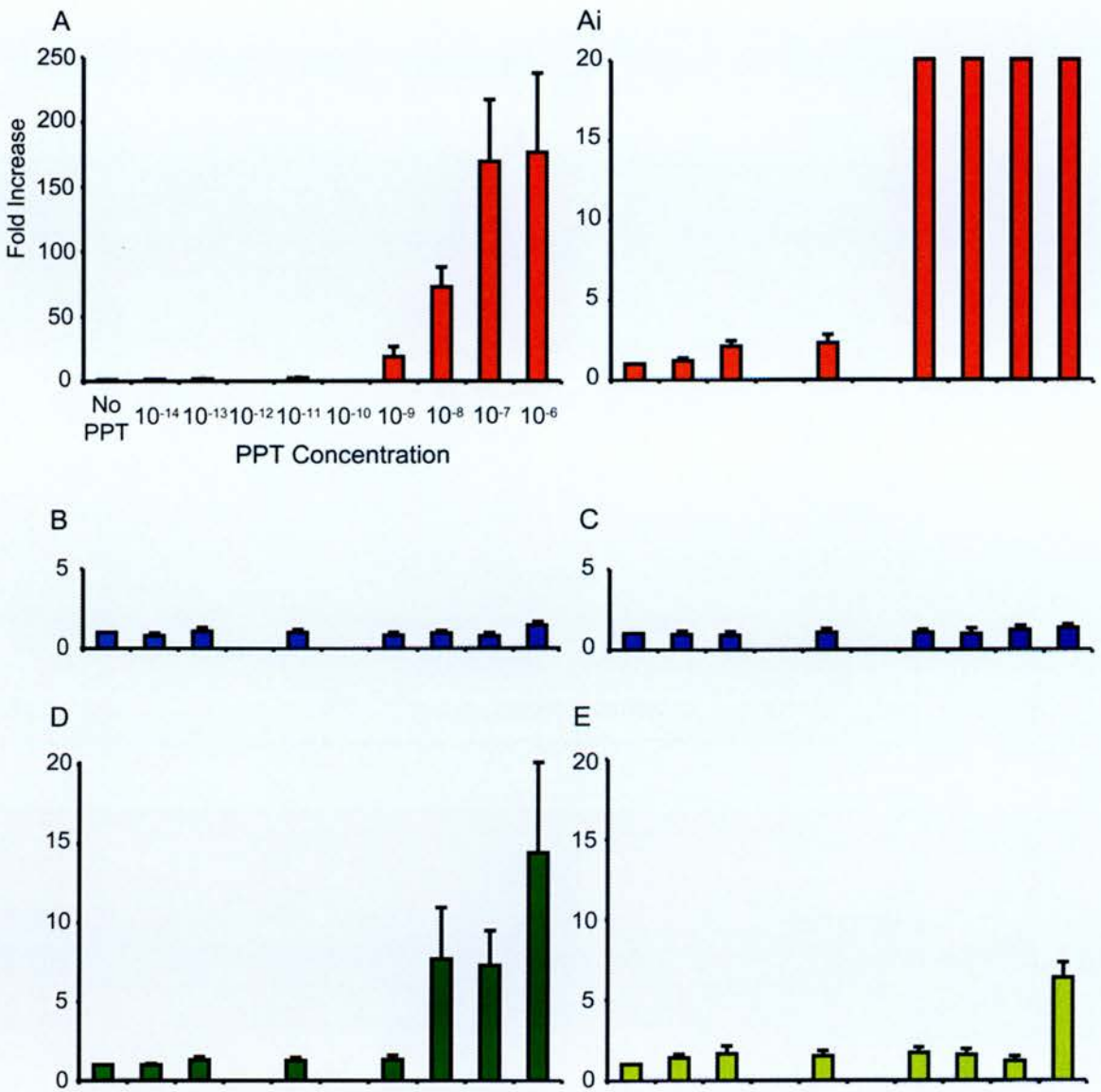


Figure 5.9. PPT™ dose response in Hep G2 cells. Hep G2 cells were transiently transfected with hERα (A), hERα; reduced scale (Ai), hERβ1 (B), hERβ2 (C), Mac ERβ1 (D) or Marm ERβ1 (E), the 3x ERE-Luc reporter and the pRL-CMV internal control. Cells were treated with a vehicle, or increasing concentrations of PPT™, ranging from 10^{-14}M to 10^{-6}M. Data are displayed as fold induction of luciferase activity over the un-stimulated cell. Data are the mean +/- SEM of 3 experiments.

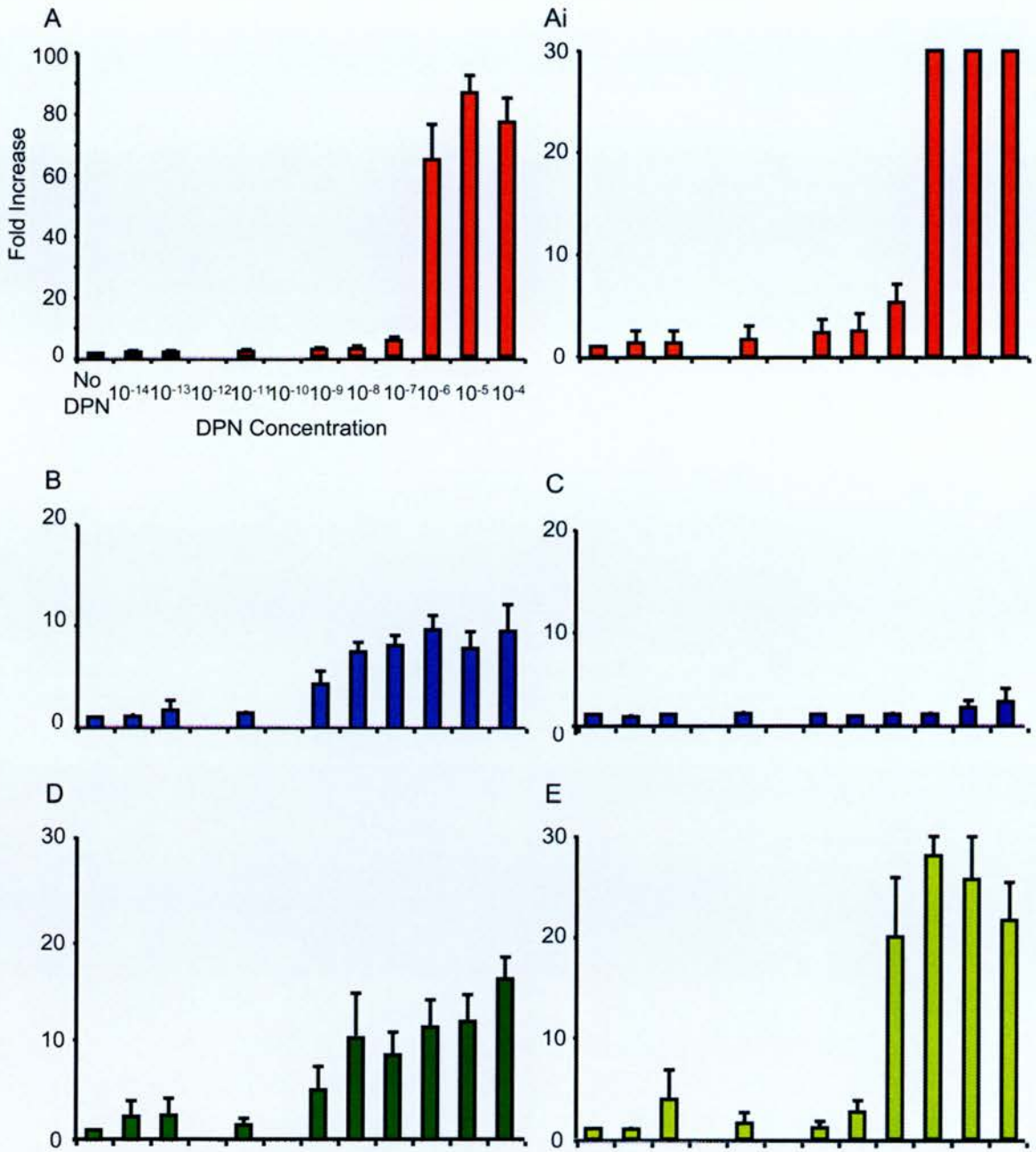


Figure 5.10. DPN™ dose response in Hep G2 cells. Hep G2 cells were transiently transfected with hERα (A), hERα; reduced scale (Ai), hERβ1 (B), hERβ2 (C), Mac ERβ1 (D) or Marm ERβ1 (E), the 3x ERE-Luc reporter and the pRL-CMV internal control. Cells were treated with a vehicle, or increasing concentrations of DPN™, ranging from 10^{-14}M to 10^{-4}M. Data are displayed as fold induction of luciferase activity over the un-stimulated cell. Data are the mean +/- SEM of 3 experiments.

5.3.2.6 Summary of Results using Single Transfections and Different Ligands

The transcriptional activity of hERα, hERβ1 and hERβ2 in the presence of oestrogenic ligands was receptor subtype dependent in both Hek 293 cells and Hep G2 cells and transcriptional activity depended upon the ligand added (*Tables 5.4* & *5.5*). Highest levels of reporter gene activation were observed using hERα in Hep G2 cells, the most marked induction was achieved using the ERα-selective ligand PPT™ (169-fold using 10^{-7}M). However, E_2 stimulated the greatest level of transcriptional activation overall through the 3x ERE reporter with ERα and ERβ1. ERβ2 failed to stimulate ERE-Luc reporter gene activation with any concentration of the ligands tested.

Table 5.4. Summary of reporter gene activation in Hek 293 cells with E_2 and 3βAdiol.

ER	10^{-8}M E_2	10^{-7}M E_2	10^{-8}M Adiol	10^{-7}M Adiol
hERα	22.1	30.3	13.6	14.0
hERβ1	9.7	11.1	13.3	17.6
McERβ1	6.2	16.8	14.5	14.7
MmERβ1	11.3	15.2	8.3	9.8

Table 5.5. Summary of reporter gene activation in Hep G2 cells with different ligands.

ER	10^{-8}M E_2	10^{-7}M E_2	10^{-8}M Adiol	10^{-7}M Adiol	10^{-8}M Ge	10^{-7}M Ge	10^{-8}M PPT™	10^{-7}M PPT™	10^{-8}M DPN™	10^{-7}M DPN™
hERα	31.5	59.4	4.4	67.6	2.3	3.7	72.7	169.1	2.5	5.3
hERβ1	8.5	11.7	2.5	5.4	1.9	4.9	1.0	1.2	7.5	7.8
McERβ1	5.8	12.1	8.8	12.2	3.2	2.8	7.6	7.1	10.8	9.1
MmERβ1	8.9	17.1	1.2	5.5	1.0	4.8	1.6	1.2	2.6	20.1

5.3.3 ERα and ERβ Co-transfections

Results presented in the previous sections have demonstrated reporter gene activation by ERα or ERβ is not functionally equivalent and is influenced by the concentration of E_2. The impact of heterodimer formation on reporter gene activation was investigated by co-transfection of variable amounts of ERβ1 or ERβ2 together with a fixed amount of ERα or ERβ1 and 3x ERE-Luc reporter. The amount of hERβ1 or hERβ2 were chosen to give final ratios of 1:1, 1:3 or 1:6. All results were compared to those obtained in control transfections involving either ERα alone plus the 3x ERE-Luc reporter (*Figure 5.11A* & *B* & *Figure 5.12A* & *B*) or ERβ1 alone plus the 3x ERE-Luc reporter (*Figure 5.11C* & *Figure 5.12C*).

5.3.3.1 Co-transfections of ERα and ERβ1

In Hek 293 cells, co-expression of ERα/ERβ1 resulted in increased activation of the ERE-Luc reporter compared to the activity seen in ERα containing controls (*Figure 5.11A*). The effect was independent of the ratio of ERα:ERβ1 although highest levels of transcriptional activity were obtained with ERα/ERβ1 (1:3) using 10^{-7}M E_2. Results obtained using Hep G2 cells were not identical (*Figure 5.12A*) and addition of ERβ1 to ERα-containing cells failed to enhance reporter gene activation. At ratios of 1:6 (ERα/ERβ1) some blunting of the transcription response was observed (see *Table 5.6*).

5.3.3.2 Co-transfection of ERα and ERβ2

In Hek 293 cells, co-expression of ERα/ERβ2 resulted in a decrease in activation of the ERE-Luc reporter activity compared to when ERα was singly transfected with the ERE-Luc (*Figure 5.11B*) with 10^{-9}M and 10^{-7}M E_2 present. The observed decrease in activation was similar with all concentrations of ERβ2 co-transfected, although with 1:3 and 1:6 ratios (ERα:ERβ2) the rate of error increased. In Hep G2 cells a definite decrease in reporter gene activation was observed upon co-transfection of ERβ2 (*Figure 5.12B*). The substantial decrease in activation was consistent with all three ratios of ERβ2 (1:1, 1:3, 1:6). Although it was noted that in the absence of E_2, the co-transfected ERα/ERβ2 increased ERE reporter gene activation compared to ERα alone in both cell lines.

5.3.3.3 Co-transfection of ERα and ERβ2

Co-transfection of ERβ1 and ERβ2 in Hek 293 cells resulted in an increase in ERE reporter gene activation (*Figure 5.11C*). The increase in reporter activation with addition of 10^{-9}M and 10^{-7}M E_2 was shown to increase as the concentration of transfected ERβ2 increased. In Hep G2 cells reporter gene activation also increased upon co-transfection of ERβ2, increasing with increasing concentrations of ERβ2 (*Figure 5.12C*). Overall, the increase in reporter gene activation in the co-transfections was greater in Hek 293 cell compared to Hep G2 cells.

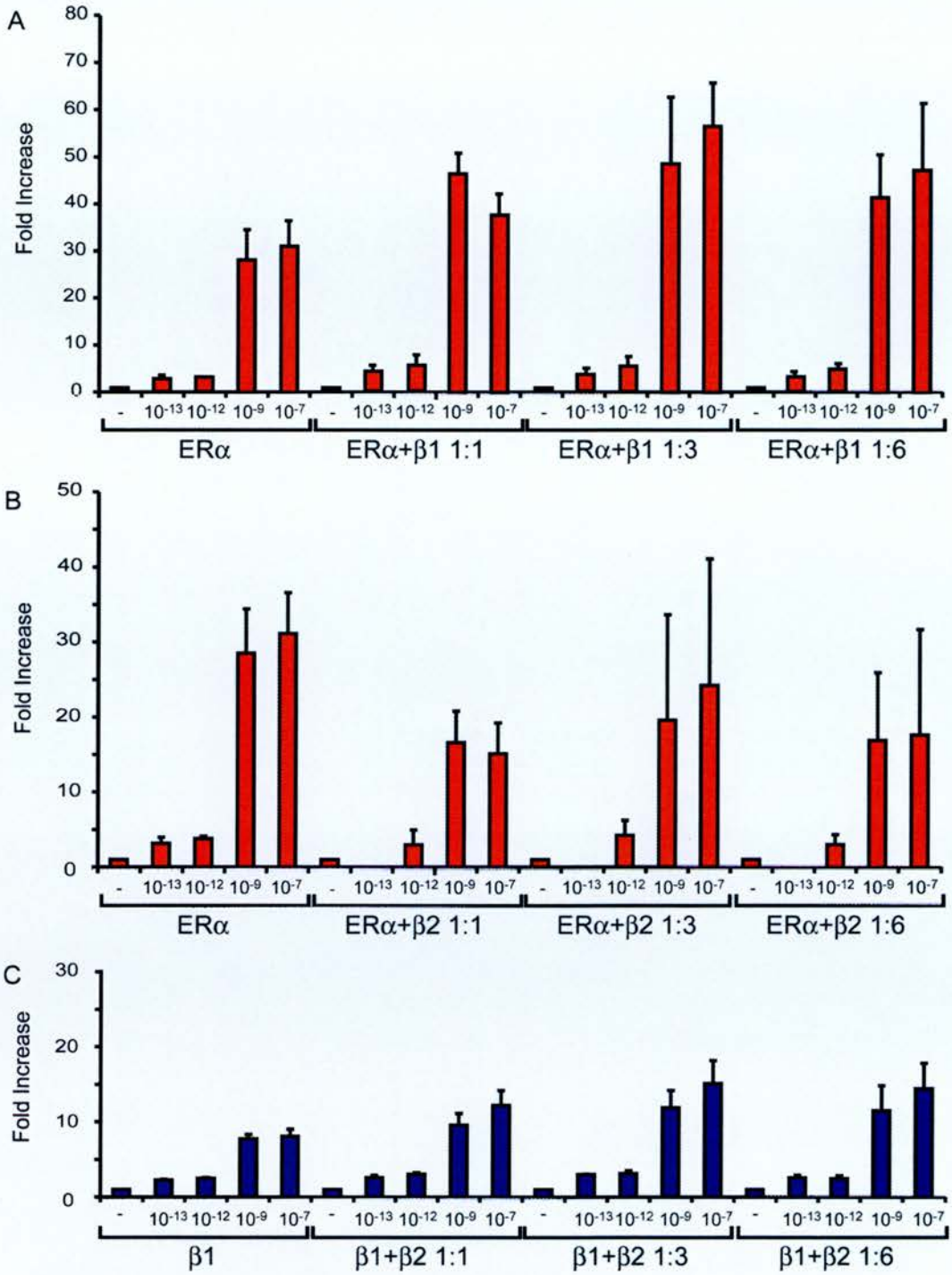


Figure 5.11. The impact of co-transfecting different ERs in Hek 293 cells. Hek 293 cells were transiently transfected using the Gene Porter transfection reagent, with the 3x ERE-Luc reporter and pRL-CMV internal control. Human ERα (A & B) was transfected into the cells together with increasing amounts of hERβ1 (A) or hERβ2 (B). hERβ1 was transfected into the cells (C) and co-transfected with hERβ2. Cells were treated with a vehicle, or increasing concentrations of E_2, ranging from 10^{-13}M to 10^{-7}M. Data are displayed as fold induction of luciferase activity over the un-stimulated cell, scales presented are different. Data are the mean +/- SEM of 3 experiments.

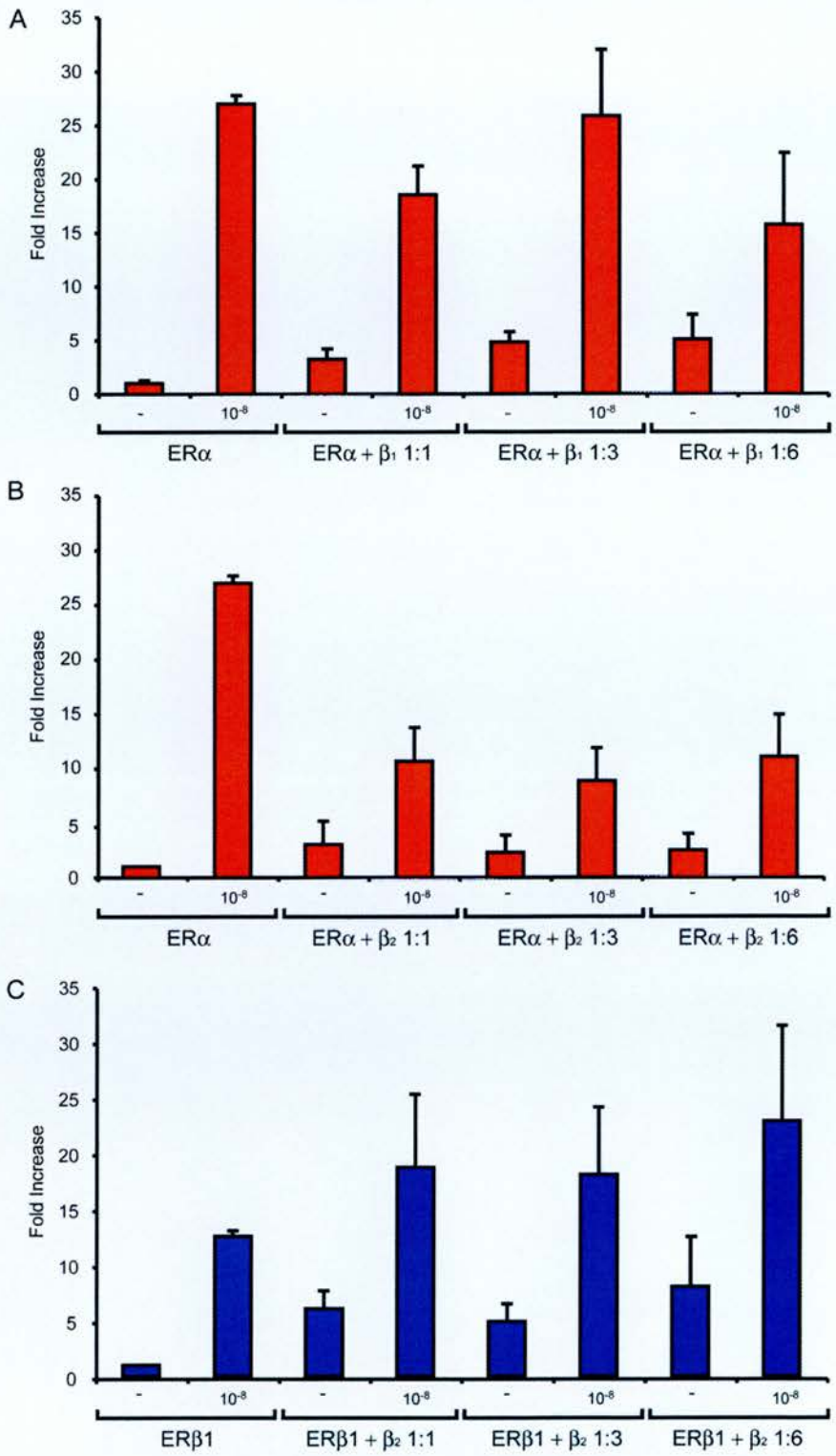


Figure 5.12. The impact of co-transfecting different ERs in Hep G2 cells. Hep G2 cells were transiently transfected with the 3x ERE-Luc reporter and pRL-CMV internal control. Human ERα (A & B) was transfected into the cells together with increasing amounts of hERβ1 (A) or hERβ2 (B). hERβ1 was transfected into the cells (C) and co-transfected with hERβ2. Cells were treated with a vehicle, or 10^{-8}M E_2. Data are displayed as fold induction of luciferase activity over the un-stimulated cell. Data are the mean +/- SEM of 3 experiments.

5.3.3.4 Summary of Results using Co-transfections

The results of the co-transfection studies in Hek 293 and Hep G2 cells are summarised in the tables below (*Tables 5.6* & *5.7*). The Hek 293 cells co-transfected with ERβ-containing constructs differed in transcriptional response compared to Hep G2 cells. Hek 293 cells showed an increase in ERE reporter gene transcription with ERα/ERβ1 and ERβ1/ERβ2 co-transfected but a decrease in gene activation with ERα/ERβ2. The Hep G2 cells demonstrated a decrease in reporter gene activation with ERα/ERβ1 and a larger decrease with ERα/ERβ2, however an increase in gene activation was observed with ERβ1/ERβ2 co-transfected.

Table 5.6. Summary of the reporter gene activation when ERs were co-transfectioned (1:6 ratio) in Hek 293 cells.

3x ERE	+	+	+	+	+	+	+	+	+	+
ERα	+	+	+	+	+	+	-	-	-	-
ERβ1	-	-	+	+	-	-	+	+	+	+
ERβ2	-	-	-	-	+	+	-	-	+	+
10^{-7}M E_2	-	+	-	+	-	+	-	+	-	+
Response	1.0	31.2	1.1	47.1	1.1	17.6	1.0	8.1	1.2	14.4

-/+ = construct absent/present, response values given as the mean fold increase.

Table 5.7. Summary of the reporter gene activation when ERs were co-transfectioned (1:6 ratio) in Hep G2 cells.

3x ERE	+	+	+	+	+	+	+	+	+	+
ERα	+	+	+	+	+	+	-	-	-	-
ERβ1	-	-	+	+	-	-	+	+	+	+
ERβ2	-	-	-	-	+	+	-	-	+	+
10^{-8}M E_2	-	+	-	+	-	+	-	+	-	+
Response	1.0	26.8	5.5	15.8	2.5	10.9	1.0	12.7	7.9	22.3

-/+ = construct absent/present, response values given as the mean fold increase.

5.3.4 Impact of SRC-1 on Transcriptional Activity of hERα, hERβ1 or hERβ2

5.3.4.1 Impact of SRC-1e and SRC-1a in Co-transfections with ERα and ERβ

The impact of SRC-1e and SRC-1a on the transcriptional activity of human ERs was assessed using transient transfections in Hep G2 cells (*Figure 1.13*). Transcriptional activation of the 3x ERE-Luc reporter by ERα stimulated by addition of 10^{-7}M E_2 was increased by SRC-1a but decreased by SRC-1e compared to the activity of ERα alone. With lower concentrations of E_2 (10^{-8}M) both SRC-1e and SRC-1a enhanced transcriptional activity of hERα when compared to levels of reporter gene expression induced by hERα without SRC-1.

Human ERβ1 co-transfected with SRC-1e or SRC-1a resulted in a slight decrease in reporter gene activation by E_2 compared to when hERβ1 was transfected without SRC-1 (*Table 5.8*). However the overall levels of reporter gene activation were very low.

The human ERβ2 transfected into Hep G2 cells did not induce any reporter gene activity in the presence of E_2 and co-transfection of SRC-1e or SRC-1a did not result in any change in activity.

Table 5.8. Summary of reporter gene activity in Hep G2 cells when co-transfecting SRC-1e and SRC-1a with ER.

ER	No SRC		SRC-1e		SRC-1a	
	10^{-8}M	10^{-7}M	10^{-8}M	10^{-7}M	10^{-8}M	10^{-7}M
hERα	4.9	55.3	6.5	16.9	14.8	64.4
hERβ1	1.5	3.4	1.0	1.3	1.4	2.3

Values given as the mean fold increase.

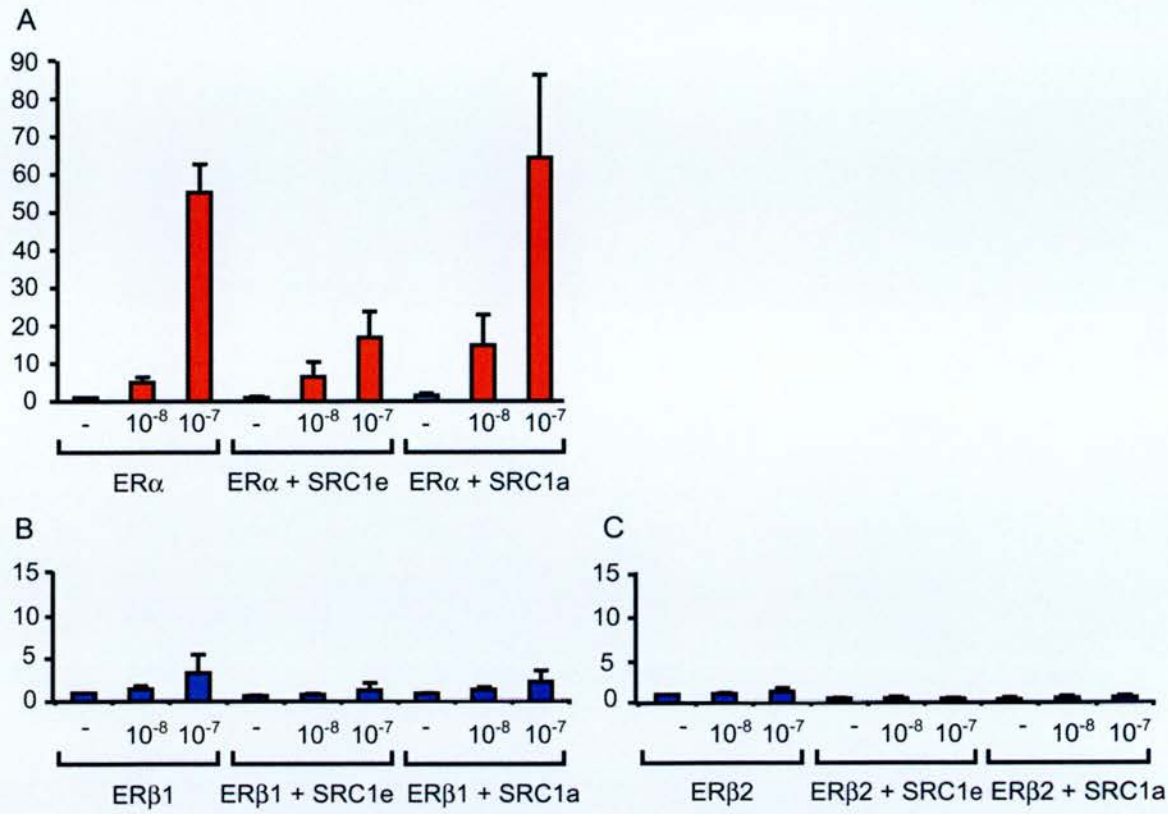


Figure 5.13. Impact of SRC1 co-transfected with hERα, hERβ1 or hERβ2 in Hep G2 cells. Hep G2 cells were transiently transfected with hERα (A), hERβ1 (B) or hERβ2 (C), the 3x ERE-Luc reporter and pRL-CMV internal control. Cells were treated with either a vehicle, E_2 at 10^{-7}M or 10^{-8}M. Data are displayed as fold induction of luciferase activity over the un-stimulated cell. Data are the mean +/- SEM of 3 experiments.

5.3.4.2 Impact of the Presence of SRC-1e in ERα and ERβ Co-transfections

Co-transfection studies (section 5.3.3 and *Figure 5.14*) using Hep G2 cells demonstrated that co-expression of hERβ1 or hERβ2 together with hERα resulted in an apparent reduction in ERE-Luc reporter gene activity with 10^{-8}M E_2. In contrast, co-expressing hERβ2 with hERβ1 increased activity compared to hERβ1 alone. Co-expression of SRC-1e with ERα alone was found to reduced ERE reporter activity. However when SRC-1e was co-transfected with ERα and ERβ1, reporter gene activation increased to greater than the level of activation of ERα alone with the 3x ERE-Luc reporter construct or with ERα and ERβ1. When the ERα and ERβ2-containing constructs were co-transfected the ERE-Luc reporter gene activation decreased compared to ERα and 3x ERE-Luc alone, and the presence of SRC-1e did not act to alter the transcriptional activation of the reporter. In co-transfections with ERβ1 and ERβ2 reporter gene activation increased with increasing concentrations of ERβ2. In the presence of SRC-1e the reporter gene activation increased further (as shown in *Table 5.9*).

Table 5.9. Summary of the effect of SRC-1e co-transfected with the hERα, hERβ1 and hERβ2 and the ERs co-transfected in a 1:6 ratio, on reporter gene activation in Hep G2 cells.

ERE	+	+	+	+	+	+	+	+	+	+	+
ERα	+	+	+	+	+	+	+				
ERβ1				+	+			+	+	+	+
ERβ2						+	+			+	+
SRC-1e			+		+		+		+		+
10^{-8}M E_2		+	+	+	+	+	+	+	+	+	+
Response	1	26.1	19.5	18.7	33.6	14.9	20.4	5.1	5.2	5.3	11.1

-/+ = construct absent/present, response values given as the mean fold increase.

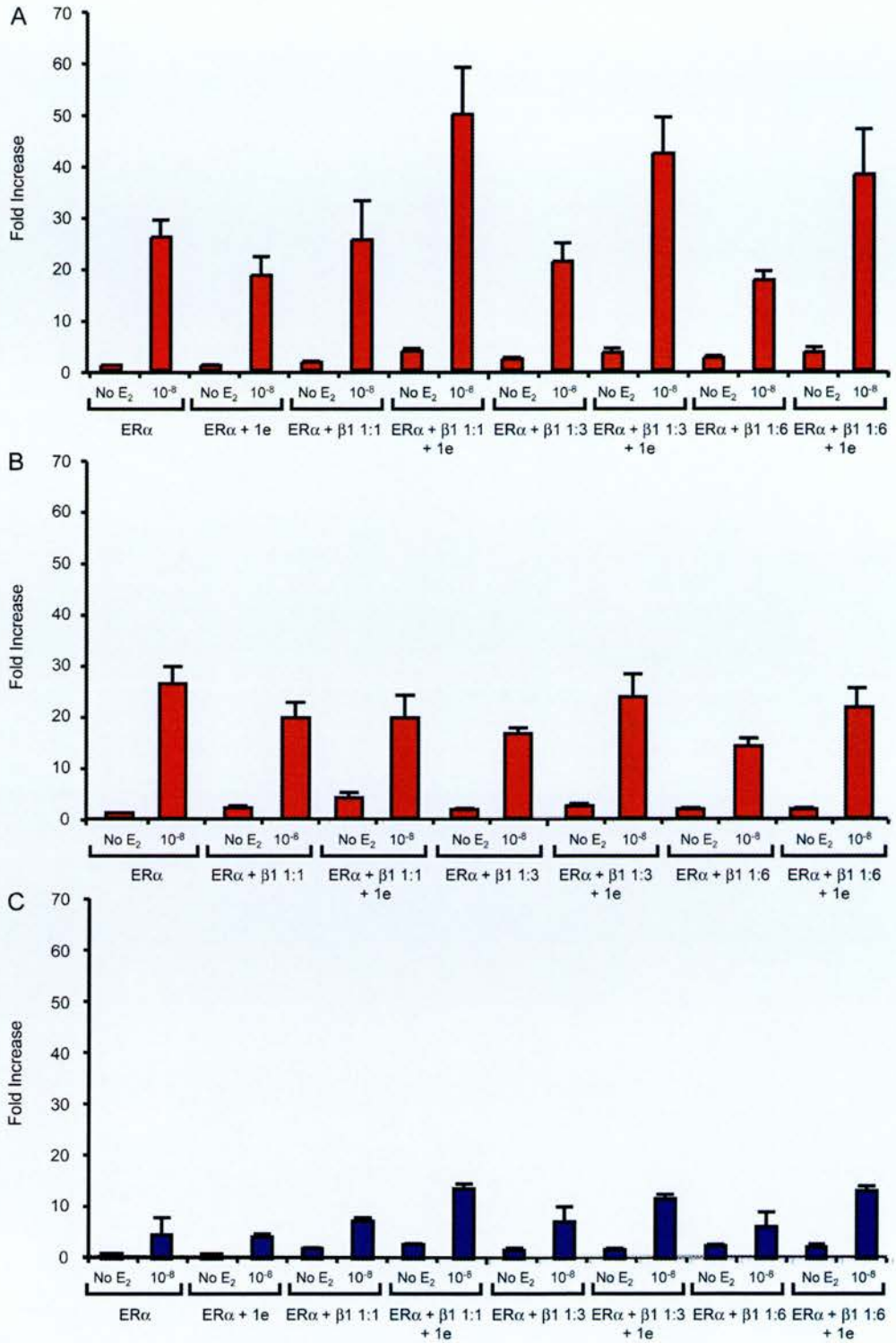


Figure 5.14. Impact of SRC1e with hERα, hERβ1 and hERβ2 co-transfections in Hep G2 cells. Hep G2 cells were transiently transfected with hERα and hERβ1 (A), hERα and hERβ2 (B) or hERβ1 and hERβ2 (C), the 3x ERE-Luc reporter and pRL-CMV internal control. Cells were treated with a vehicle, or 10^{-8}M E_2. Data are displayed as fold induction of luciferase activity over the un-stimulated cell. Data are the mean +/- SEM of 3 experiments.

5.4 Discussion

These functional studies with different ER constructs, ERE reporter genes and different ligands demonstrate the variability of this complex system. In these experiments regardless of the cell type or ERE sequence, human ERα is a more potent activator of ERE reporter gene transcription than ERβ1 (wild type). The ERβ2 variant did not initiate gene transcription regardless of ligand, reporter gene or cell type. Cells were transiently transfected with the same concentrations of ERα and ERβ1, however the level of expression in indvidual cells cannot be controlled.

Studies have compared the functional activity of ERβ from human as well as primate and compared this with human ERα. The impact of different ligand concentrations, promoter gene sequence, and the presence of a co-activator (SRC-1) have been explored using transient transfections in two ER negative cell lines (Hek 293 and Hep G2). Preliminary data has been gathered assessing the impact of receptor co-transfection (heterodimer formation) on reporter gene activation.

5.4.1 Activation of ERα and ERβ1 by 17β-Oestradiol is Influenced by the Promoter

Ligand-activated oestrogen receptors bind with high affinity to specific DNA sequences, known as oestrogen response elements (EREs) located within the regulatory regions of target genes. Sequence-specific interaction between oestrogen receptors and the EREs is dependent upon the presence of an intact DBD (Hall *et al.*, 2002; Tremblay *et al.*, 1997). However, regions outside the DBD may also play a role in formation of the stable receptor-DNA complexes (Kuiper *et al.*, 1997).

It has been reported that one base change in an ERE half site can cause the re-arrangement of a localised hydrogen bond network rather than a simple hydrogen bond substitution and therefore result in lower affinity of ERα for the imperfect EREs, as shown in CHO cells (Loven *et al.*, 2001). The ERE-bound ERα or ERβ proteins are thought to interpret the sequence and orientation of ERE half-sites in unique manners. One consequence of variation in sequence between different EREs will therefore be to induce distinct conformations in the ER co-activator pocket leading to different co-factor recruitment and gene response (Loven *et al.*, 2001).

These differences provide a mechanism for differential gene expression by ERα and ERβ1 through the classical ERE pathway.

The OT ERE is a bovine oxytocin response element mutated to resemble a rat ERE. It has one base change from the consensus vitellogenin ERE in the 5' half site which can affect its affinity for ERs and therefore its ability to activate gene transcription (Loven *et al.*, 2001). In Hep G2 cells transfected with hERα or hERβ1, addition of E_2 only resulted in 7.5- and 1.4-fold increase respectively, with OT ERE. Hek 293 cells transfected with ERβ1 showed a 1.1-fold increase in reporter gene activation with OT ERE, in contrast ERα transfected with OT ERE resulted in a 26-fold increase in reporter gene activity. This may be due to cell-specific factors in Hek 293 cells which aid OT ERE reporter gene activation via ERα.

My results demonstrate that in general the single consensus Vit EREs cloned into the pGL2 and pTAL vectors induced a greater fold increase in gene transcription than the OT ERE with ERα in Hep G2 cells. The one exception was when ERα was expressed in Hek 293 cells where a higher level of gene transcription was induced by the OT ERE reporter. The work shown here is in agreement with previous studies which demonstrate that the consensus Vit ERE induces gene transcription though hERα and hERβ1 to a greater extent that the OT ERE in Hela and CHO cells (Loven *et al.*, 2001; Wood *et al.*, 2001). This is also consistent with reports that hERα's binding affinity for Vit ERE is higher than that of hERβ1 (Loven *et al.*, 2001; Wood *et al.*, 2001) shown by DNase I footprinting.

The single consensus vitellogenin EREs did not stimulate transcriptional activation as much as the 3x ERE. These results are in agreement with previous published work using ER-negative CHO-K1 and MCF-7 cells transfected with different ERα and ERβ1 constructs including human ERs (Klinge, 1999; Sathya *et al.*, 1997; Tyulmenkov *et al.*, 2000). Changes in binding affinity of ERs in response to the increase in number of tandem EREs has revealed both similarities and differences between ERα and ERβ1. A mammalian ERα construct induced activation of luciferase reporter gene activity in response to E_2 from three or four tandem copies of a consensus ERE in CHO cells but not with two tandem copies. The same was true for ERβ1 but to a lower level of activation (Tyulmenkov *et al.*, 2000). In electrophoretic mobility shift assays mammalian ERα binds to three ERE copies

with high affinity closely followed by 4 copies, however with one or two copies of the ERE the binding affinity was significantly less. The binding affinities for mammalian ERβ1 demonstrated that it bound with comparable affinity to two and four tandem copies of ERE and with higher affinity to the three copies of the ERE. In the presence of more than four Vit EREs linked together, the reporter activity of hERα decreased reporter activity when assessed in MCF-7 cells (Nagel *et al.*, 2001). Therefore, to summarise the published studies, the relative induction of reporter gene activity by E_2 was dependent on the number of tandem copies of the consensus ERE, with three being optimal.

Ogawa *et al* have used gel shift assays to demonstrate that homodimers of human ERα or ERβ1 bind to the consensus Vit ERE with high affinity but that ERβ2/cx homodimers do not bind as effectively (Ogawa *et al.*, 1998c). Klinge has reviewed the available data on ERE binding and concluded that there was no significant correlation between ERE binding and E_2-induced transcriptional activation (Klinge, 2001). This suggests that differences in binding affinity assays may not be of direct relevance to the differences in transcriptional activation of ERα and ERβ1 with the different response elements.

In my studies I found hERα and hERβ stimulated transcriptional activation to varying extents depending upon the different EREs used and that this also varied between different cell lines. In general hERα-induced activation was greater than observed using ERβ1. This has been documented previously using different EREs in several cell lines including Cos 1, CHO and Hela (Cowley and Parker, 1999; Tremblay *et al.*, 1997; Tyulmenkov *et al.*, 2000). However, in human osteosarcoma or human endometrial carcinoma cells, the transcriptional activity of ERβ1 was higher than that of ERα (Watanabe *et al.*, 1997). Cell-specific variation in the expression of ERα and ERβ1 suggests the individual receptors may regulate specific subsets of oestrogen responsive genes in different cell backgrounds. Therefore, differences in ligand binding, tissue distribution and transcriptional activation of reporter constructs indicate that ERα and ERβ play overlapping but different roles *in vivo* (Kuiper and Gustafsson, 1997; Massaad *et al.*, 1998).

The 3x ERE reporter was the reporter chosen to study oestrogen regulated gene expression in these experiments as it was demonstrated to initiate the greatest transcriptional activity. However it is important to remember that most endogenous

genes contain EREs that differ from the consensus Vit ERE by one or more base pairs and that these differences in nucleotide sequence most likely influence the ability of individual EREs to mediate transcription activation.

5.4.2 ERα and ERβ1 Contribute Differentially to Gene Activation by Oestrogenic Ligands

Functional studies described in this chapter demonstrate that ligands such as 17β-oestradiol initiate gene transcription with human ERα and ERβ1 (wild type) but not with the ERβ2 variant. The ERβ2 variant lacks the last coding exon which includes amino acids involved in ligand binding and all of those involved in the AF-2 domain and is therefore unable to bind ligands (Kuiper *et al.*, 1996; Ogawa *et al.*, 1998c). These studies have also shown for the first time that macaque ERβ1 and marmoset ERβ1 are able to initiate transcription of the 3x ERE reporter using a range of ligands. ERα activated ERE reporter gene transcription more than ERβ1 with all ligands tested, except with the ERβ1-specific agonist DPN™. It was also noted that the maximal stimulation was observed at different concentrations of ligand with the different constructs, with ERβ1 generally requiring a higher concentration of ligand for maximal transcription to occur compared to the hERα.

The idea that ligands play an active role in ER function has been proposed by several laboratories (McDonnell *et al.*, 1995; Paige *et al.*, 1999; Wijayaratne *et al.*, 1999). It has been demonstrated that different ligands induce distinct conformational changes in the ER, correlating with diversity in transcriptional responses (Hall and Korach, 2002; Hall *et al.*, 2002). It has been demonstrated that upon binding, different ligands induce distinct conformational changes within the receptor co-activator-binding pockets, thus leading to differential co-factor recruitment and biological activity (Hall *et al.*, 2002). This agrees with my data which demonstrates that different oestrogenic ligands induce different levels of transcriptional activity even when the same ERE promoter is used.

The variation in ligand-induced activation shown in these results is also in agreement with previous observations in which ERβ1 consistently induced reporter gene activity to a lesser extent than ERα (Barkhem *et al.*, 1998; Pettersson *et al.*, 2000). My results have demonstrated that ERβ1 is poorly activated by E_2 at concentrations below 10^{-9}M (1nM). In contrast, ERα reaches approximately 66% of

its maximal activation with 10^{-9}M E_2 in Hek 293 cells but only 20% in Hep G2 cells with the same E_2 concentration. However, previous saturation ligand binding assay studies using rat ERs have demonstrated that there is very little difference in the ERα and ERβ1's binding affinity for E_2 (Kuiper *et al.*, 1997).

To my knowledge, transient transfections have not been performed previously to investigate ER-induced transcription in the presence of 3βAdiol. In my studies 3βAdiol was generally found to activate gene transcription via ERβ1 to a greater extent than ERα, however there were differences observed between Hek 293 and Hep G2 cells. Kuiper *et al* studied the ligand specificities of rodent ERα and ERβ to calculate their relative binding affinities and demonstrated that 3βAdiol binds with higher affinity to ERβ than ERα and that the K_i (nM) was three times greater for ERα (6nM compared to 2nM) (Kuiper *et al.*, 1997). No data has been presented on the ER transcriptional activity in response to addition of 3βAdiol, but as it has been stated that this natural agonist serves as an agonist through ERβ1 in the prostate (Weihua *et al.*, 2002). High concentrations (10^{-7}M) of 3βAdiol resulted in an increase in gene activation of over 67-fold compared to 4.4-fold at 10^{-6}M, when ERα was expressed in Hep G2 cells. This was not observed in Hek 293 cells, nor with ERβ1 in either cell line tested. This dramatic increase in gene transcription may be due to the ERα ligand binding pocket becoming saturated and therefore over stimulating co-activator recruitment, although further work is required to determine the exact explanation.

Genistein was observed to activate gene transcription via both ERα and ERβ1 in Hep G2 cells. At higher concentrations (10^{-7}M) genistein stimulated ERβ1 gene transcription to a greater extent than ERα. Various studies have measured the binding affinity of different ERs for genistein with different values being obtained (An *et al.*, 2001; Barkhem *et al.*, 1998; Harris *et al.*, 2002a; Kuiper *et al.*, 1997). Despite binding assays generally showing genistein has a more than 10-fold higher selectivity for ERβ1 versus ERα, it has been demonstrated by crystallographic studies that genistein exhibits partial agonism on the ERβ1 LBD and full agonism on ERα LBD (Pike *et al.*, 1999). Previous studies have found that the EC_{50} of ERα for genistein is much higher than for ERβ1, indicating that ERβ1 requires less genistein in the system to activate gene transcription (Harris *et al.*, 2002b; Kuiper *et al.*, 1997). Therefore from my transfection results and previous published binding affinity data,

it can be suggested that genistein binds to ERβ1 and activates transcription through an ERE to a greater extent than when it is bound to ERα.

Pettersson *et al* also demonstrated that genistein was a more effective activator of mouse ERβ1 than human ERα in transient transfections with a 2x ERE in Hek 293 cells, however, at higher concentrations of ligand (200nM) ERβ1 and ERα were both fully active (Pettersson *et al.*, 2000). With a very high concentration (2μM) of genistein, the ligand acted as a super agonist for both ER subtypes, resulting in more than 200% of the maximal activity obtained with E_2 (Pettersson *et al.*, 2000). The high transcriptional activity reported by Pettersson *et al* may be due to genistein modulating protein kinase signalling pathways and therefore altering the phosphorylation status of ERs. However, this high fold increase was not observed in the present studies, nor was it observed by Barkhem *et al* who used stably transfected Hek 293 cells and incubated them with genistein for 72 hours (Barkhem *et al.*, 1998).

In these functional studies the novel ligand PPT™, was found to activate ERα-induced gene transcription to a much greater extent than E_2, whereas human ERβ1 did not activate transcription in the presence of PPT™. Similar results have been reported previously; Kraichely *et al* demonstrated that PPT™ induced a high level of transcriptional activation, similar to E_2, with ERα using a 3x ERE-CAT reporter (Kraichely *et al.*, 2000). However, PPT™ was not found to stimulate ERβ1 with a range of reporter gene constructs even at high concentrations (10μM) of ligand (Kraichely *et al.*, 2000). In contrast, for the first time it has been demonstrated that macaque and marmoset ERβ1 constructs were able to activate the 3x ERE reporter in the presence of high concentrations of PPT™ (10^{-8}M and 10^{-6}M respectively). This indicates that there may be slight differences in the nucleotide sequence within the LBD of the primate ERβ1 which allows binding of PPT™ in a manner which can initiate gene transcription, unlike the human ERβ1.

The ERβ-specific agonist DPN™ induced gene transcription through ERα to a much higher extent than via ERβ1 at high concentrations (10^{-6}M and greater). This was unexpected as it has been proposed that DPN™ is an ERβ1-specific agonist and is 170-fold more potent with ERβ1 compared to ERα with a 4x ERE in HEC-1 cells (Meyers *et al.*, 2001). In a recent study, Ramsey *et al* demonstrated in CHO-KI cells

with a 2x ERE, that DPN™ activated hERβ1 induced transcription to a greater extent than hERα at a range of concentrations (from 10^{-11} to 10^{-4}M) and hERα only became transcriptionally activated at 10^{-8}M DPN™ (Ramsey *et al.*, 2003). However in my functional studies, ERβ1 was more transcriptionally active than hERα only at lower concentrations of DPN™ (10^{-7}M) where ERα was minimally active. The dramatic increase in transcriptional activity of ERα in the presence of high concentrations (10^{-6}M) of ligand may be due to saturation of the receptor's LBD pocket and recruitment of co-activators. Marmoset ERβ1 induced higher levels of gene transcription through a 3x ERE with all concentrations of DPN™ tested, compared to macaque ERβ1 or human ERβ1, highlighting a possible difference between different species and the ER conformation once the ligand has bound.

5.4.3 The Effect of Co-transfecting Two ER Isoforms

In Chapter 4 it was observed that FP-tagged human ERα, ERβ1 and ERβ2 can heterodimerise within the cell nucleus and the dose response experiments (this chapter) have shown that hERα and hERβ1 are not functionally equivalent and ERβ2 is not functional in the presence of ligand. Therefore functional studies were necessary to ascertain whether hERβ1 or hERβ2 could reduce hERα's transcriptional activity by acting as a transdominant repressor or a modulator for hERα action.

Experiments in Hek 293 cells with the 3x ERE reporter and 10^{-8}M E_2 have shown that co-transfection of hERα with hERβ1, or hERβ1 with hERβ2 resulted in an increase in transcriptional activity compared with transfections of hERα or hERβ1 alone. However, when hERα was co-transfected with hERβ2, the fold increase in transcriptional activity was decreased slightly. However, these results must be considered preliminary as there were variations between experiments. This variation in responsiveness is most likely due to the transiently transfected constructs not being introduced into every cell, as observed in Chapter 3 using immunocytochemistry. Therefore in the co-transfections not all the cells will express both of the receptors.

In Hep G2 cells co-transfecting hERβ1 with hERα and a 3x ERE reporter, resulted in a decrease of gene transcription but the results were variable and depended on the different ratios of hERα:ERβ1. When hERβ2 was co-transfected with hERα, gene

transcription levels were decreased substantially. However when hERβ1 and hERβ2 were co-expressed transcriptional activity was increased. In the absence of E_2, when hERβ1 and hERβ2 were co-expressed, a slight increase in reporter gene activation was observed. Hence indicating the possibility of an alternative, non-classical pathway for which gene transcription maybe activated through hERβ2, as this effect was not observed with hERβ1 alone. Therefore in these cells, hERβ2 may act as a transdominant repressor of hERα transcriptional activity in the presence of 10^{-8}M E_2, but may alternatively act to increase the activity of hERβ1.

When Ogawa *et al* co-transfected human ERα or hERβ1 with hERβcx (hERβ2) and an ERE-CAT reporter in Cos 7 cells at a 1:10 ratio, hERβ2 inhibited ligand induced transactivation by hERα, but did not influence transactivation by hERβ1 (Ogawa *et al.*, 1998c). They obtained similar results in Hek 293 cells. The results presented here only demonstrate this in Hep G2 cells using a 1:6 ratio, as in the Hek 293 cells there was a high error rate. Thus highlighting the differences between cell lines and variations in conditions and reporters used in different studies.

Hall *et al* have used Hek 293 cells and the same 3x ERE reporter gene to demonstrate that hERβ1 (wild type) had the ability to act as a transcriptional inhibitor or activator of hERα depending upon the agonist concentration (Hall and McDonnell, 1999). In their studies they suggested that hERβ1 functions as a repressor of hERα transcriptional activity at low, subsaturating concentrations of E_2 (10^{-10}M) and that the presence of hERβ1 decreased the sensitivity of hERα-expressing cells to oestradiol (Hall and McDonnell, 1999). However they only used a 1:1 ratio of the ERs, whereas I used a range of ratios (1:1, 1:3 & 1:6). In saturating conditions of E_2 (10^{-7}M) transcriptional activity of the ERα/ERβ1 heterodimer is indistinguishable from that of the hERα homodimer. Therefore the ability of hERβ1 to switch from a transcriptional repressor to an activator as E_2 levels increase may provide cells expressing hERα and hERβ1 with a mechanism to control cellular sensitivity to hormones (Hall and McDonnell, 1999). Pettersson *et al* also demonstrated that ERβ can act as a negative or positive regulator of ER activity depending upon the concentration of ligand added (Pettersson *et al.*, 2000), stating that ERβ inhibits ERα activity at E_2 concentrations below the ERβ activity threshold. However they used human ERα and a mouse ERβ (short form) in Hek 293 cells with a 2x ERE reporter, therefore a direct comparision cannot be made with my results.

In the results shown here, 10^{-8}M and 10^{-7}M E_2 was used in Hek 293 cells and 10^{-7}M in Hep G2 cells, which Hall *et al* and Pettersson *et al* found to be saturating. If a lower concentration of ligand had been used a different result may have been obtained.

However the hERβ1, hERβ2 and hERβ5 variants have all been reported to inhibit transcriptional activation by hERα using an ERE promoter in Cos 1 and Cos 7 cells. The hERβ1, hERβ2 and hERβ5 variants appeared to have different efficiencies; hERβ1>hERβ2>hERβ5 (Peng *et al.*, 2003). Peng *et al* demonstrated a decrease in activation of transcription via hERα when it was co-transfected with hERβ1 or hERβ2 in a 1:9 ratio occurred with both 10^{-10}M and 10^{-7}M E_2 (Peng *et al.*, 2003). This contradicts what Hall *et al* demonstrated with subsaturating and saturating E_2 concentrations (Hall and McDonnell, 1999). This recently published data by Peng *et al* shows both similarities and differences to my data, as I demonstrated that hERβ1 acted to decrease hERα transcriptional activity in Hep G2 cells, but slightly increased activity in Hek 293 cells.

Inoue *et al* studied an ERβ splice variant (ERβΔ5) which lacked exon 5 (Inoue *et al.*, 2000). ERβΔ5 has both an intact DNA binding domain and an AF-1 transactivational domain, therefore being similar to ERβ1, but due to a premature stop codon lacks the LBD present in exon 5. The ERβΔ5 isoform was found to have a dominant negative effect on ERβ1 or ERα in transient transfection systems (Inoue *et al.*, 2000). One possible mechanism for the documented dominant negative activity is that these heterodimers are transcriptionally inactive and have no or very little DNA binding activity. The other possibility is that the ERβΔ5 competes with ERα or ERβ1 for the DNA binding site and forms transcriptionally inactive heterodimers on the ERE (Inoue *et al.*, 2000). In the process of transactivation by ER, AF-1 and AF-2 need to interact with co-activators. Since ERβΔ5 lacks the AF-2 but contains an intact AF-1, unproductive interactions with co-activators may be maintained, but the interaction of co-activators with wild-type ER may be prevented (Inoue *et al.*, 2000). These data are highly relevant to ERβ2 and may explain one way in which ERβ2 can act as a dominant negative "receptor".

In my studies I have found that co-transfections of hERβ1 and hERβ2 resulted in an increase of ERE reporter activity compared to when hERβ1 was present alone in the

Hek 293 and Hep G2 cells. This was similar to data published previously by Ogawa *et al* who found that in Cos 7 cells with a single ERE reporter the level of transcriptional activity was maintained at the same level as hERβ1 in the absence of ERβ2 (Ogawa *et al.*, 1998c). The observed increase in transcriptional activity in my results may be due to cell specific factors in the human cell lines that increase ERβ1 activity and/or act to stimulate ERβ2 in an alternative manner. There is also the possibility that ERβ2 acts through a novel and as yet unidentified pathway through more specific response elements, phosphorylation or novel binding.

It has been recently proposed that the difference in hERα and hERβ1 activity may be due to cell-type specific factors as well as the slight differences in the LBD (Ramsey *et al.*, 2003). ERβ1 contains an N-terminal AF-1 region which is less active than ERα's AF-1 and its activity is thought to be cell-specific. This hypothesis of cell specificity is based on the greater activity of ERα AF-1 in Hep G2 cells whereas in Hek 293 cells AF-1 and AF-2 activity are equal. Therefore in Hek 293 cells the N-terminus of hERβ1 has greater transcriptional activity than in Hep G2 cells. If this is true, it may explain why in my experiments ERα transcriptional activity is 6-fold greater than ERβ1 with 10^{-7}M E_2 in Hep G2 cells, but only 3-fold greater in Hek 293 cells. This may also explain why in co-transfections in Hek 293 cell ERα and ERβ1 show an increase in transcriptional activity whereas the same co-transfection in Hep G2 cells shows a decrease in activation.

There are several hypotheses as to how ERβ1 may repress ERα induced reporter activity in co-expression studies, including (i) ERβ1 binds to cognate response elements in a constitutive manner and therefore competes with the ERα for access to DNA (Hall and McDonnell, 1999); (ii) ERβ1 does not contain a strong AF-1 within its amino-terminus, but rather contains a repressor domain that when removed increases overall transcriptional activity (Hall and McDonnell, 1999; Ogawa *et al.*, 1998b); (iii) with subsaturating concentrations of E_2, inactive ERβ1 binds to its ERE and competitively blocks ERα binding to the ERE. As the E_2 levels increase, sufficient ERβ1 is activated to compete with the unligand bound ERβ1 and transcription can proceed (Hall and McDonnell, 1999; Pettersson *et al.*, 2000); (iv) modulatory effects on the configuration of the AF-2/AF-1 interaction, where ERβ may be repressing the AF-1 activity (Pettersson *et al.*, 2000) and (v) the nuclear receptors compete via transcriptional silencing or squelching (Schodin *et al.*, 1995),

for example, titrating out co-activators and basal transcription factors essential for ERα. These hypotheses have previously been based on studies using ERα and aim to explain the possible dominant negative of ERβ1, however since the identification of ERβ splice variants, such as ERβ2 (Ogawa *et al.*, 1998c) these statements may also apply to ERβ2 containing heterodimers.

It has previously been determined that human ERα/ERβ1 heterodimers are formed preferentially over ERβ1/ERβ2 heterodimers in gel shifts (Ogawa *et al.*, 1998c). And ERα homodimers are formed preferentially in comparison with ERβ homodimers (Cowley *et al.*, 1997). ERα homodimers and ERα/ERβ1 heterodimers were found to bind to the ERE with greater affinity than ERβ1 homodimers (~4-fold greater K_d) (Cowley *et al.*, 1997) suggesting the former dimers are more functional than the latter. Both ERα and ERβ have been detected in many tissues by RT-PCR (Cowley *et al.*, 1997; Moore *et al.*, 1998) and co-expression has been demonstrated by immunohistochemistry (Saunders *et al.*, 1998; Saunders *et al.*, 2000). Recently data have been extended to include expression of the ERβ2 splice variant (Chapter 3 and (Saunders *et al.*, 2002a; Scobie *et al.*, 2002), however the relative amounts of ER proteins in specific cell types has yet to be determined. Therefore in tissues varying levels of expression of ERs, and the presence of splice variants will result in different homo- and heterodimers forming.

5.4.4 The Ability of the SRC-1 to Alter ERα or ERβ1 Transcriptional Activation in the Presence of E_2

In this study the SRC-1 co-activator was co-transfected into the Hep G2 cells together with a human ER. In the presence of E_2 and it was found that the SRC-1 isoforms had a variable impact on gene transcription. At 10^{-8}M E_2, the SRC-1e and SRC-1a co-activators increased human ERα's ability to initiate gene transcription, however when 10^{-7}M E_2 was added to the cells, SRC-1e inhibited hERα's activity and SRC-1a increased the activity compared to hERα without the co-activator. Human ERβ1's transcriptional activity was slightly decreased in the presence of SRC-1e and SRC-1a. Therefore these results are variable with different concentrations of ligands, hence more work is required to determine the precise response of ERs with SRC-1 transfected into the cell. The hERβ2 variant did not activate gene transcription either with or without the SRC co-activators.

The SRC-1a and SRC-1e isoforms not only differ in their ER-binding properties but also in their ability to potentiate the transcriptional activity of the human ER in transiently transfected Cos 1 cells (Kalkhoven *et al.*, 1998). The extra LXXLL motif in the SRC-1a isoform increases the affinity for the ER *in vitro* but it does not appear to be responsible for the functional difference between the two isoforms (Kalkhoven *et al.*, 1998). This difference has been proposed to be due to a second activation domain (AD2) that is CBP independent and is suppressed in the SRC-1a isoform. Therefore SRC-1 exists as functionally distinct isoforms which are likely to play different roles in ER-mediated transcription (Kalkhoven *et al.*, 1998). However, this effect was found to be cell and promoter dependent. SRC-1e stimulated ER mediated transcription on various reporters with SRC-1a only enhancing activity on an ERE-tk-Luc reporter (Kalkhoven *et al.*, 1998) not with a 3x ERE as used in my experiments. The inability of SRC-1a to potentate the transcriptional activity of the ER on simple reporters and the oxytocin reporter suggests that on some promoters the recruitment of CBP/p300 is not sufficient for maximal ER-mediated transcription in transiently transfected cells (Kalkhoven *et al.*, 1998). However, it has been documented that there are cell differences in transient transfection studies, for example; Cos 7 cells showed a greater stimulation by SRC-1e than HeLa cells (Kalkhoven *et al.*, 1998). In the current experiments using Hep G2 cells, SRC-1a stimulated hERα transcriptional activation more than SRC-1e, however, with hERβ1 there was very little difference between the results obtained in the presence of the two co-activator isoforms. To support this, recently Ramsey *et al* have demonstrated in CHO-KI cells with a 2x ERE, that SRC-1 can increase hERα transcriptional activation but not hERβ1 (Ramsey *et al.*, 2003).

Co-transfection of SRC-1 and ERβ1 has previously been shown to increase the basal level of transcriptional activation of an ERE reporter even in the absence of oestrogenic ligand in Cos 1 cells (Kalkhoven *et al.*, 1998; Tremblay and Giguere, 2001) consistent with a ligand-independent effect of SRC-1 on ERβ1. However, the basal level of transcriptional activation was not increased when SRC-1 was added to mouse ERα in the absence of ligand (Tremblay and Giguere, 2001). This finding was observed in the current study but only in the co-transfection studies in which ERα and ERβ were co-transfected and SRC-1e was present.

In the absence of ligand it has been demonstrated that ERα and SRC-1 both tagged to different FPs, are diffusely distributed within the nucleus of MCF-7 cells (Stenoien *et al.*, 2000). Addition of E_2 results in a substantial overlap between the two proteins therefore suggesting that a significant portion of the SRC-1 is associated with ERα. In the cells examined, E_2 caused the SRC-1 to become punctate (focal) and overlap with the distribution of ERα, whereas in the absence of ERα or presence of antagonists, SRC-1 did not redistribute. Stenoien *et al* have also demonstrated that the ER-SRC interaction system is very dynamic (Stenoien *et al.*, 2001b). Photobleaching studies (FRAP) show that in the absence of ligand ER and SRC-1 have a rapid complex exchange, in the presence of E_2 the interactions are less transient and the exchange rate is reduced but remains highly dynamic.

Ligand binding is known to induce conformational changes that reveal interaction surfaces required for SRC-1 binding and different ligands bound to ERα or ERβ1 are able to induce differential recruitment of co-activators (Hall *et al.*, 2002). Structural studies provide insight into the importance of helix 12 for co-activator interactions. The E_2 bound ER LBD exists in a specific conformation which is different to the conformation when other ligands are bound (Stenoien *et al.*, 2001b). One difference is the position of helix 12 which forms a surface that is important for AF-2 activity after binding agonists. In particular the co-activator NR box lies in the groove formed by the agonist-induced re-positioning of helix 12 (Shiau *et al.*, 1998). These published studies provide a structural mechanism for the differential effects of agonist and antagonists and highlight that re-arrangements involving helix 12 are very important for ER activity and interactions with co-activators. Deletion experiments have shown the importance of helix 12 in the ER's re-organisation and therefore ER's function (Shiau *et al.*, 1998; Stenoien *et al.*, 2001b). Weatherman *et al* used fluorescence resonance energy transfer (FRET) to determine that the interaction of ERα with LXXLL-containing peptides was dependent on the AF-2 domain. And that the interaction, although very weak, could occur in the absence of ligand but was dramatically stabilised in the presence of ligand (Weatherman *et al.*, 2002).

Kraichely *et al* used GST pull-down and yeast two-hybrid assays to determine the differences in the relative strength of interaction of human ERα and hERβ1 with SRC-1 in the presence of agonist ligands, they suggested that these differences

may prove to be physiologically important and contribute to selective activities depending on the expression levels of the co-activator in particular cell types (Kraichely *et al.*, 2000). Human ERβ1 interacts with SRCs with lower affinities compared to hERα (Kraichely *et al.*, 2000; Wong *et al.*, 2001), therefore giving a possible reason for it's lower transcriptional activity.

In this study it has been demonstrated that the presence of SRC-1e with co-transfections of hERα with hERβ1 or with hERβ2, increased transcriptional activity and opposed any dominant negative effect observed with hERβ2. The presence of SRC-1e increased the reporter gene activation with hERα and hERβ1, and ERα with ERβ2, to a greater extent than that of hERα alone. Co-transfections with hERβ1 and hERβ2 showed the transcriptional response was increased in the presence of SRC-1e. This observed increase in reporter gene transcription despite the presence of the hERβ may be due to the cells becoming saturated with SRC-1. It has been proposed that the decrease in reporter gene transcription when ERβ forms heterodimers is possibly due to a decrease in ability of the dimer to recruit co-activators (Peng *et al.*, 2003). Therefore in a system such as the one demonstrated, over expression of the SRC-1 aids transcription. It was also shown that when the hERβ variants were co-transfected with SRC-1e in the absence of E_2 transcriptional activity increased, further supporting the theory of an alternative activation mechanism.

In these studies, RT-PCR with primers specific for SRC-1a and SRC-1e detected both mRNAs in Hek 293 and Hep G2 cell lines (data not shown). Needham *et al* have also demonstrated that mRNAs for SRC-1a and SRC-1e was expressed in these cell lines, but at a ratio of 1:2. However their relative protein concentration is still unknown (Needham *et al.*, 2000).

5.4.6 Conclusions

My findings, alongside other published data, suggest that in human tissues activation of oestrogen-responsive genes will be influenced by the tissue-specific expression of ERα and/or the ERβ1 wild type and/or ERβ2 variant, the ligand present, the affinity of the receptor for individual ERE sequences, ligand and ERE-induced changes in the receptor conformation, the presentation of transcriptionally productive surfaces for interaction with other transcription factors and the

contribution of numerous transactivating factors in the promoters of oestrogen responsive genes. This level of complexity enables cells with these receptors to exert exquisitely fine-tuned control over cellular functions in response to ligands. Due to these complex interactions it is very difficult to generalise for a certain receptor or a certain ligand what the response will be *in vivo*, therefore careful consideration is required when applying results such as these to a more physiological system.

Chapter 6

Impact of Activation of the MAP kinase Pathway on Oestrogen Receptors

6.1 Introduction

It has been demonstrated in previous chapters that the different oestrogen receptors can co-localise within the nucleus of the same cell (Chapter 4 and Stenoien *et al.*, 2000) and functional studies have shown that the receptor isoforms have different transcriptional activation potential with different ligands (Chapter 5 and Hall and Korach, 2002). Sequence analysis has revealed conservation of some amino acids within the ligand binding domain and DNA binding domains of ERα and ERβ1. Both receptors bind to oestrogenic ligands, with some affinity differences (Kuiper *et al.*, 1998) and activate gene transcription to fairly similar extents. However, the ERβ2 splice variant is truncated at the C-terminus and therefore lacks the specific amino acids which encode for the AF-2 region and hence cannot be activated by ligands (Pettersson and Gustafsson, 2001). My functional studies have demonstrated that hERβ2 can have a dominant negative effect on hERα, but acts to increase gene activation when co-transfected with hERβ1 in Hek 293 and Hep G2 cells. Similar results have been recently documented in Cos 1 and Cos 7 cells with an ERE reporter (Peng *et al.*, 2003). It has also been observed that in the absence of E_2, hERβ2 sometimes shows an increase in gene transcription when present with hERβ1 and/or SRC-1e. Therefore my results have lead to the suggestion that hERβ2 is activated through an alternate, ligand-independent mechanism.

6.1.1 AF-1 Domain and Phosphorylation

ERα and ERβ possess a constitutive N-terminal activation function (AF-1) whose activity can be modulated by kinase signalling pathways. A serine residue (Ser118) in the A/B domain of human ERα has been shown to mediate epidermal growth factor signalling through the Ras/MAPK signalling cascade (Kato *et al.*, 1995). However, Ser118 of ERα does not seem to be involved in the recruitment of co-activators (Webb *et al.*, 1998). It has been demonstrated that the mouse ERβ1 can also be activated by the Ras pathway and this effect is mediated through a Serine residue (Ser124) in the A/B domain (Tremblay *et al.*, 1997). More recently it has

been demonstrated ERβ contains two Serine residues within the AF-1 region. These serine residues positioned at 87 and 106, are involved in the phosphorylation of the receptor by MAP kinase (Driggers *et al.*, 2001; Rochette-Egly, 2003). It is also within the AF-1 region of ERβ that upon ER phosphorylation, co-activators such as SRC-1 can be recruited (Tremblay *et al.*, 1999a).

6.1.2 Growth Factor Stimulation

Accumulating evidence has demonstrated significant cross-communication between oestrogen receptors and peptide growth factor signalling pathways with some reports suggesting that growth factors may promote activation of ERs even in the absence of natural ligands (Marquez *et al.*, 2001). Agents capable of exerting such ligand independent activation of ER include epidermal growth factor (EGF) (Ignar-Trowbridge *et al.*, 1996; Kato *et al.*, 1995; Kato *et al.*, 1998) and insulin-like growth factor (Ignar-Trowbridge *et al.*, 1996; Kato *et al.*, 1998; Lee *et al.*, 1997).

In the absence of oestrogens *in vivo* administration of EGF mimics the effects of oestrogen on the mouse reproductive tract (Curtis *et al.*, 1996; Ignar-Trowbridge *et al.*, 1996). In mice lacking ERα expression (αERKO) both oestrogen- and EGF-stimulated uterine growth is blocked, although the EGF signalling pathway has not been disrupted (Curtis *et al.*, 1996). In addition, EGF alone can increase the expression of the progesterone receptor in the mammary glands in an ER-dependent manner, mimicking the effects of oestradiol. Likewise, inhibition of EGF action in the mammary gland blocks oestradiol-induced expression of the progesterone receptor and development of terminal end buds (Ankrapp 1998). In EGF receptor knock out mice, the stromal but not the epithelial response to oestradiol is severely limited in both the uterus and the vagina (Hom *et al.*, 1998). The conclusion drawn from these results is that the ERs may mediate transcription of target genes by integrating signals from growth factor activated pathways as well as from ligand binding, especially in reproductive tissues.

6.1.3 Epidermal Growth Factor Receptor (EGFr) and Activation

The EGF receptor is a 170-kDa transmembrane glycoprotein that consists of an extracellular ligand-binding domain in its amino terminus, a transmembrane spanning region and a cytoplasmic EGF stimulated protein kinase in its C terminus (Marquez *et al.*, 2001). Following binding to the EGF ligand, the receptor dimerises

and undergoes transphosphorylation at discrete tyrosine residues. This provides binding sites for signalling or linker/adapter molecules that contain Src-homology 2 domains and the recruitment of additional signalling molecules (Levin, 2003). Such proteins include non-receptor tyrosine kinases such as Src, Grb or Sos members. Signalling cascades are then triggered, as pictured in *Figure 6.1*, dependent on the translocation, membrane association and activation of tyrosine, serine/threonine and lipid kinases including ras, raf, protein kinase C and phosphotidylinositol 3-kinase (PI3K). Kinases translocate to the nucleus where they phosphorylate and thereby activate transcription factors, such as ERK, that induce a variety of intermediates or effectors (Levin, 2003; Marquez *et al.*, 2001).

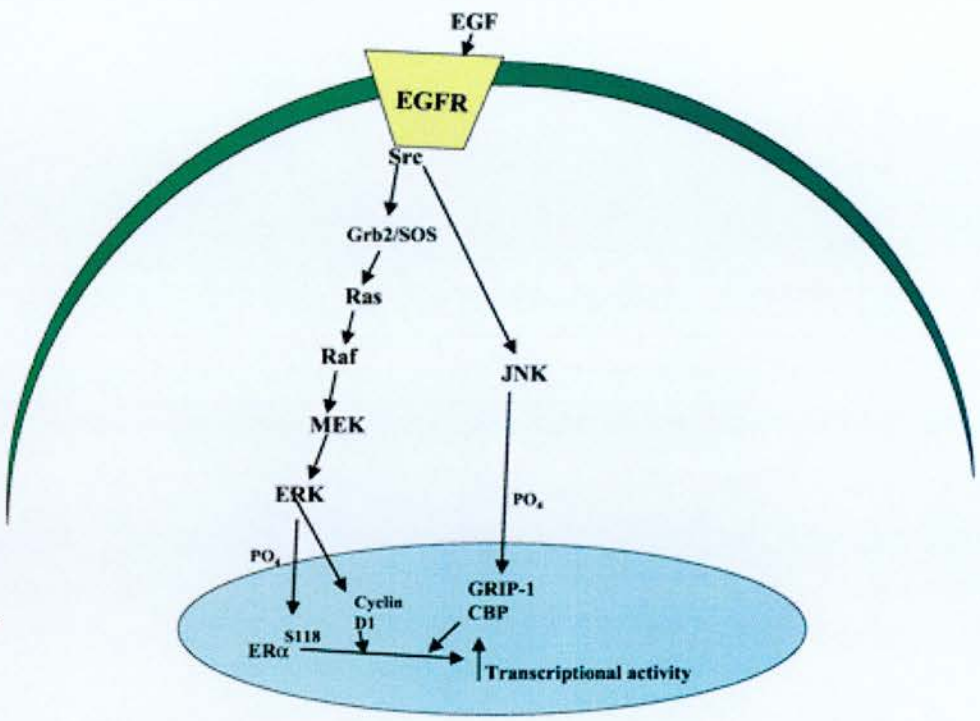


Figure 6.1. EGF activating EGF and the signalling cascade resulting in activation of ERα. Taken from Levin (2003).

6.1.4 Insulin-like Growth Factor Activation

As well as evidence for an interaction between the EGF and ER signalling pathways there is evidence indicating cross-talk between the IGF-1 system and ER. IGF-1 binding activates its receptor, leading to PI3K/AKT activation, increased ERα synthesis and augmented ERα transcriptional activity. This is suggested to result from the phosphorylation of serine residue (Ser118) in the AF-1 region (Martin *et al.*, 2000). Similar to EGF receptor activation, IGF-1 activates aspects of uterine cell

proliferation *in vivo*, and this is dependent on ERα (Klotz *et al.*, 2002). In addition E_2 can stimulate the activity of many proteins associated with the IGF-1 signalling cascade including insulin receptor substrate (IRS) proteins, IGF-I receptors, and IGF-binding proteins (Lee *et al.*, 1999). Therefore, there appears to be an important cooperation and cross-talk between these two systems.

6.1.5 Alternative ERβ2 Activation Pathways

It has been recently documented that the oestrogen receptor can become rapidly activated within seconds, which cannot be explained by the classical steroid ligand mechanism (Levin, 2003). Data has been published regarding the human ERα and mouse ERβ1 and their abilities to be phosphorylated (discussed in the literature review), however relatively nothing is understood about hERβ1 phosphorylation and there are no publications concerning phosphorylation of the ERβ2 variant. ERβ2 lacks amino acids essential for the AF-2 region and therefore cannot be activated in the classical ligand dependent manner as demonstrated for ERα and ERβ1 (shown in Chapters 4 and 5). Sequence alignment (Chapter 3) has shown that the amino acid sequences of human ERβ1 and ERβ2 within the A/B domain are identical, hence it can be assumed that ERβ2 has the same phosphorylation sites as ERβ1 in this N-terminal region. Therefore it is possible that the ERβ2 could be phosphorylated and hence be activated in the same way as ERβ1.

6.1.6 Aims

The aims of this chapter were to investigate if the ERs could be activated to stimulate gene transcription following growth factor induced phosphorylation and whether ERβ2 could be activated by this alternative pathway. It was also important to determine the localisation of the growth factor receptor and to determine whether it was present in the same cells that expressed ERβ2 so that the alternative pathway was physiologically viable.

6.2 Methods

6.2.1 Transient Transfections for Confocal Microscopy

To be able to analyse human ERα, ERβ1 and ERβ2 protein expression in living cells, the full length cDNAs were cloned into the fluorescent protein vectors (EGFP and DsRed) purchased from Clontech as described in section 2.9 and Chapter 4. Transfections were performed as described in section 2.10.2.6. Hek 293 or Hep G2 cells were plated onto 35mm glass bottom microwell dishes (Plastik® Corporation). For each dish used, 2μg DNA was transfected into the cells using JetPEI (section 2.10.2.2). After 48 hours the media was removed and the cells washed in PBS at room temperature. To maintain the cells during analysis, 1ml PBS with 25mM Hepes buffer (Sigma) was added to the dish. The epidermal growth factor used was diluted to 10^{-8}M in PBS/Hepes and added to the dish that was already in place on the warm plate (37°C) on the confocal microscope (LSM510, Zeiss). Images were captured over a period of time at 10 minute intervals for 1 hour and exported into Photoshop 7.0 (Adobe, CA).

6.2.2 Identification of EGFr and IGFr mRNA by RT-PCR

Hek 293 and Hep G2 cells were collected, total RNA extracted and oligo DT primed cDNA synthesis performed as described in section 2.3.

6.2.2.1 Screening Primers

Specific primers were chosen, as outlined in section 2.5.1, to amplify regions of the human EGF receptor and human IGF receptor cDNAs in order to be able to determine whether mRNAs with specific sequence homology were expressed in the cell lines (*Table 6.1*).

Table 6.1. PCR primers used for detection of EGFr and IGFr and the GAPDH positive control.

	5' primer	3' primer	Product size
EGFr	GTGTGCCCACTACATTGACGC	GTTGGACAGCCTTCAAGACC	237 bp
IGFr	ATGCTGTTTGAACTGATGCG	TCTCCATGTTCTCTGGCTCC	166 bp
GAPDH	CTGCACCACCAACTGCTTAGC	ATGCCAGTGAGCTTCCCGTTC	247 bp

6.2.2.2 RT-PCR

PCR was carried out using Mega Mix (Microzone) as described in section 2.5.2.2. The PCR reactions were annealed at 55°C for 30 seconds using the EGFr and IGFr oligonucleotide primers (*Table 6.1*). DNA fragments were analysed by migration on agarose gels (section 2.6.1).

6.2.3 Immunocytochemical Analysis of EGFr in Cell lines

Immunocytochemical analysis was performed on the cell lines (Hek 293 and Hep G2) that were used in the transient transfection studies. The cells were plated out as in section 2.10 onto chamber well slides (Nalgene Nunc International). The cells were left to grow for 48 hours changing the media after 24 hours.

After 48 hours the cells were fixed in ice-cold methanol (section 2.10.6.4) and immunocytochemistry performed as section 2.11, without the de-waxing and antigen retrieval stages. The cells were incubated in 3% hydrogen peroxide in methanol for 30 minutes and then blocked with Avidin/Biotin in NRS/TBS/BSA as described in section 2.12. The monoclonal anti-EGF receptor antibody (Sigma) was added at a 1:50 dilution and the cells incubated overnight at 4°C. After washing the cells, they were incubated with Envision secondary HRP complex (DAKO) for 30 minutes at room temperature. The cells were then washed and developed with DAB to visualise receptor localisation (section 2.12). The slides were counter stained with haematoxylin, dehydrated and mounted with pertex, then photographed as described in section 2.13.

6.2.4 Transient Transfections

The ERα, ERβ1 and ERβ2 constructs and the 3x ERE-Luc reporter were prepared as previously described (section 2.7). The transfections were carried out as described in section 2.10.2.3 and Chapter 5. For every experiment, each condition was performed in duplicate, and the experiment performed a minimum of three times.

JetPEI (Cambridge Biosciences) and Gene Porter (Gene Therapy Systems) transfection reagents were used for transfecting the cells (section 2.10.2) in the experiments. Using the Hek 293 and the Hep G2 cells, 1μg ER, 0.5g 3x ERE-Luc and 0.2μg pRL-CMV was used in each well of the 12 well plate. The growth factors

were added to the cells four hours after transfection. EGF and IGF were added at concentrations ranging from 10^{-14}M to $2.5x10^{-5}$M.

The Luciferase Assay (Promega) was carried out as described in Section 2.10.2.4. The results were normalised and are presented as a fold increase over the control ER.

6.2.5 EGF Activation of the MAP Kinase Pathway

6.2.5.1 Transient Transfections for Western Analysis

Cells were plated out into 60mm dishes (Cellstar®) (section 2.10.2.7) and transfected with 6μg plasmid DNA using JetPEI (section 2.10.2.2). The cells were incubated with either media containing 10% charcoal stripped fetal calf serum, or media containing 10% serum replacement (Sigma, Catalogue Number; S-9388). After 48 hours the cells were harvested using the Nuclear Extraction method (section 2.10.2.7.1.2).

6.2.5.2 Western Blot Analysis

The collected protein samples were mixed with SDS/β-mercaptoethanol, boiled and loaded onto the "SDS" polyacrylamide gel (section 2.10.2.7.3.1). Once the proteins had migrated through the gel they were blotted on an Immobilion membrane (section 2.10.2.7.3.3). After the protein had been transferred onto an Immobilon membrane the gel was Coomassie stained (section 2.10.2.7.3.4) to ensure that the proteins had transferred properly. Membranes were blocked with 5% milk/TBST as discussed in section 2.10.2.7.3.4 prior to overnight incubation with the primary antibody.

6.2.5.3 The MAP Kinase and Phosphorylated MAP Kinase Antibodies

Polyclonal antibodies directed against p44/p42 MAP kinase and phosphorylated MAP kinase, were purchased from Cell Signalling Technology. The MAP kinase p44/p42 antibody detects total (phosphorylation-state independent) p44 and p42 MAP kinase proteins without cross-reacting with JNK/SAPK or p38 MAP kinase homologues. The MAP kinase and phosphorylated MAP kinase antibodies were incubated with the membrane overnight at 4°C at a 1:1000 dilution.

The following day the membranes were washed and an HRP conjugated anti-rabbit IgG (SAPU) secondary antibody (1:5000) was incubated with the membranes for 2 hours, prior to ECL developing (section 2.10.2.7.3.4).

6.2.6 Immunohistochemical Analysis of EGFr Localisation in Tissues

Human, macaque and marmoset tissues were previously collected as described in Chapter 3. The tissues had been fixed and embedded in paraffin wax blocks and were held in an archive in the histology section at the MRC Human Reproductive Sciences Unit (Edinburgh).

Immunohistochemical studies were carried out on tissues using an EGFr specific monoclonal antibody using methods described in section 2.11. Slides were subjected to 5% hydrogen peroxide in methanol for 30 minutes at room temperature. The Avidin-Biotin blocking stage was excluded and instead the sections were blocked in NRS/TBS/BSA for 30 minutes at room temperature. The primary anti-EGFr antibody was diluted in NRS in a 1:50 dilution and added to the sections, which were then incubated overnight at 4°C.

The following day, one drop of Envision secondary complex (DAKO) was applied to the sections for 30 minutes at room temperature to amplify the signal. The sections were washed and DAB was used to develop the signal. The slides were counter stained with haematoxylin, dehydrated and mounted with pertex, then photographed as described in section 2.13.

6.3 Results

6.3.1 Confocal Microscopy

To be able to visualise ERα, ERβ1 and ERβ2 in Hek 293 and Hep G2 cells and the effect of treatment with EGF, the ERβ constructs were tagged with the EGFP and the ERα to DsRed. In Hek 293 cells the FP-tagged ERα, ERβ1 and ERβ2 were all distributed in a diffuse pattern within the nucleus of the live cell in the absence of EGF. Addition of 10^{-8}M EGF induced the receptors to redistribute into discrete foci after 60 minutes (*Figure 6.1* & *6.2a*). The intensity profiles (*Figure 6.2b*) detail the focal distribution of the ERβ2-GFP receptor in three individual cells.

In Hep G2 cells, ERα, ERβ1 (data not shown) and ERβ2 remained in a diffuse distribution in the presence of EGF, even after 60 minutes (*Figure 6.3a* & *Figure 6.3.b*).

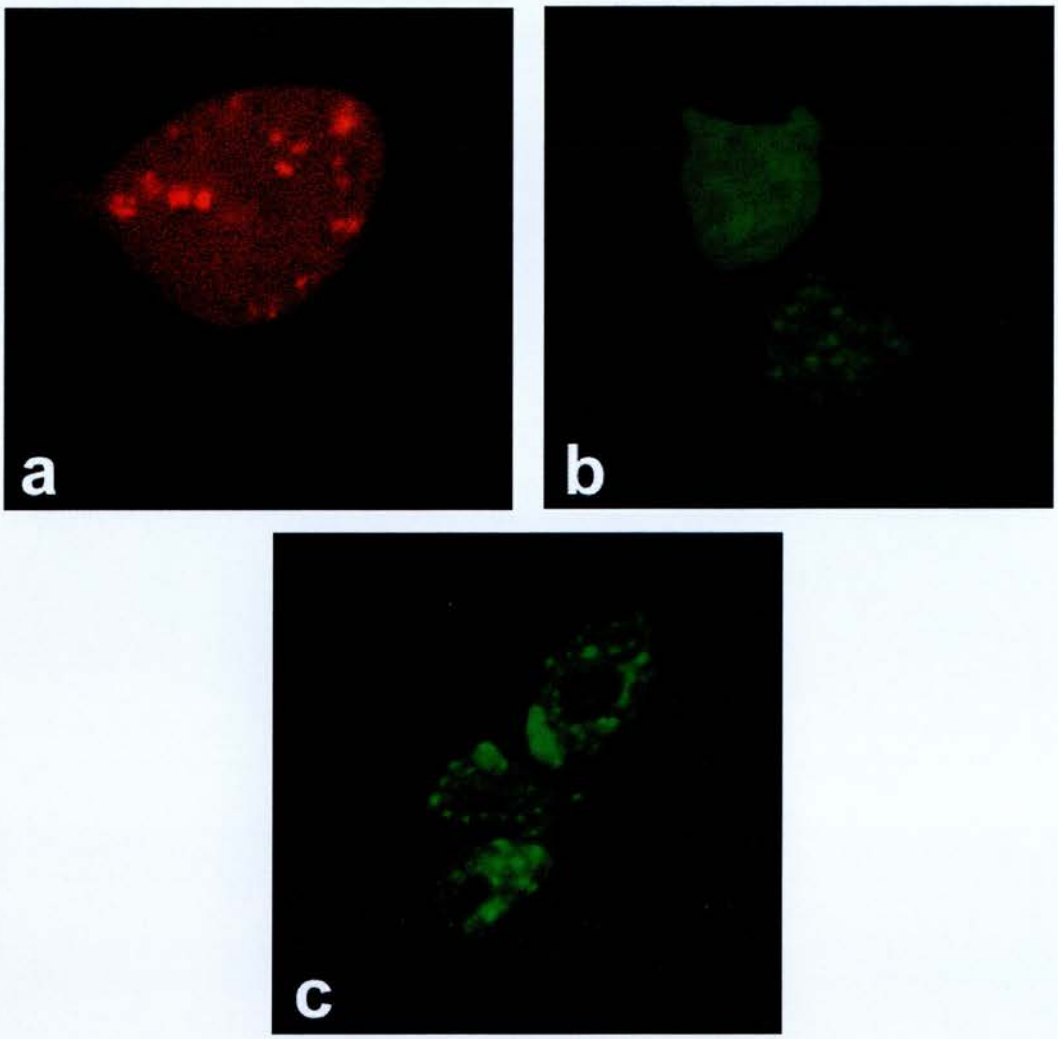


Figure 6.1. Human (a) ERα-DsRed, (b) ERβ1-EGFP and (c) ERβ2-EGFP expression in Hek 293 cells with addition of 10^{-8}M EGF. ER constructs were transiently transfected into Hek 293 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M EGF. Confocal images after 60 minutes in the presence of EGF.

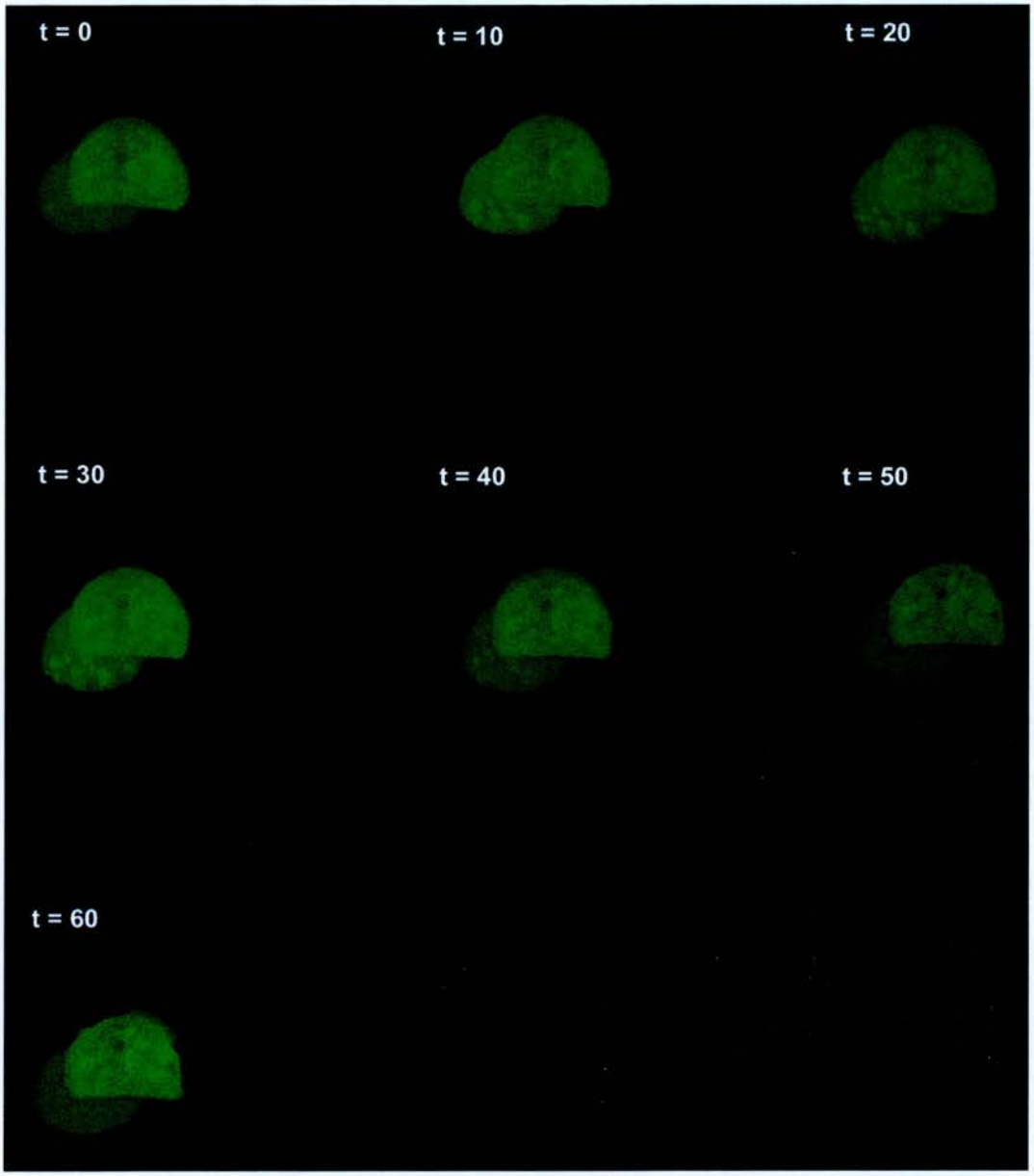


Figure 6.2a. ERβ2-EGFP expression in Hek 293 cells with addition of 10^{-8}M EGF. The ERβ2-EGFP construct transiently transfected into Hek 293 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M EGF. Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

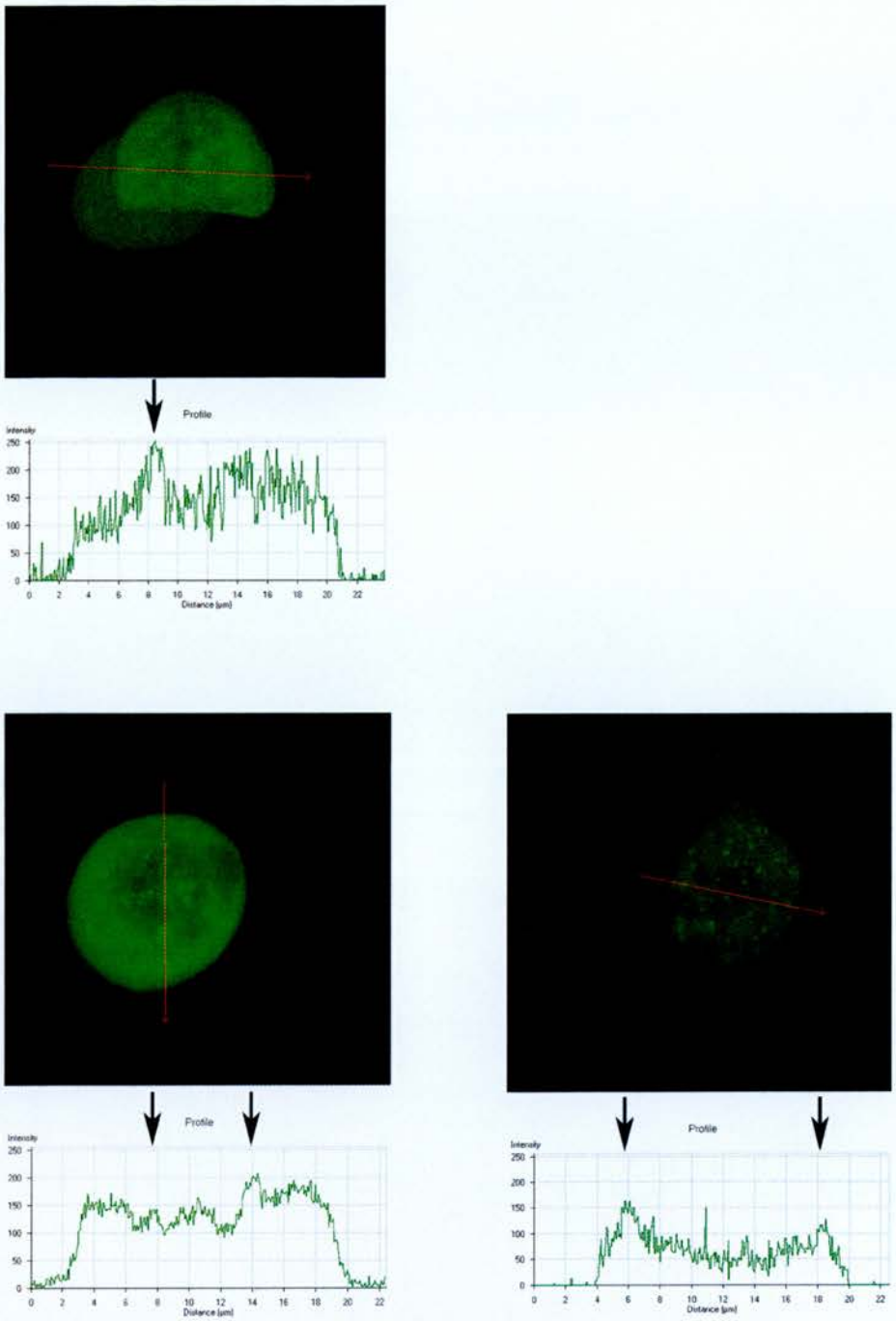


Figure 6.2b. ERβ2-EGFP expression in Hek 293 cells with addition of 10^{-8}M EGF for 60 minutes. Images are of three cell nuclei showing a graph of the intensity profile of the fluorescent ER. The graph demonstrates the intensity of the fluorescence (y axis) along the length of the line drawn through the nucleus, in the direction of the arrow (x axis). All graphs shown have the same y axis values.

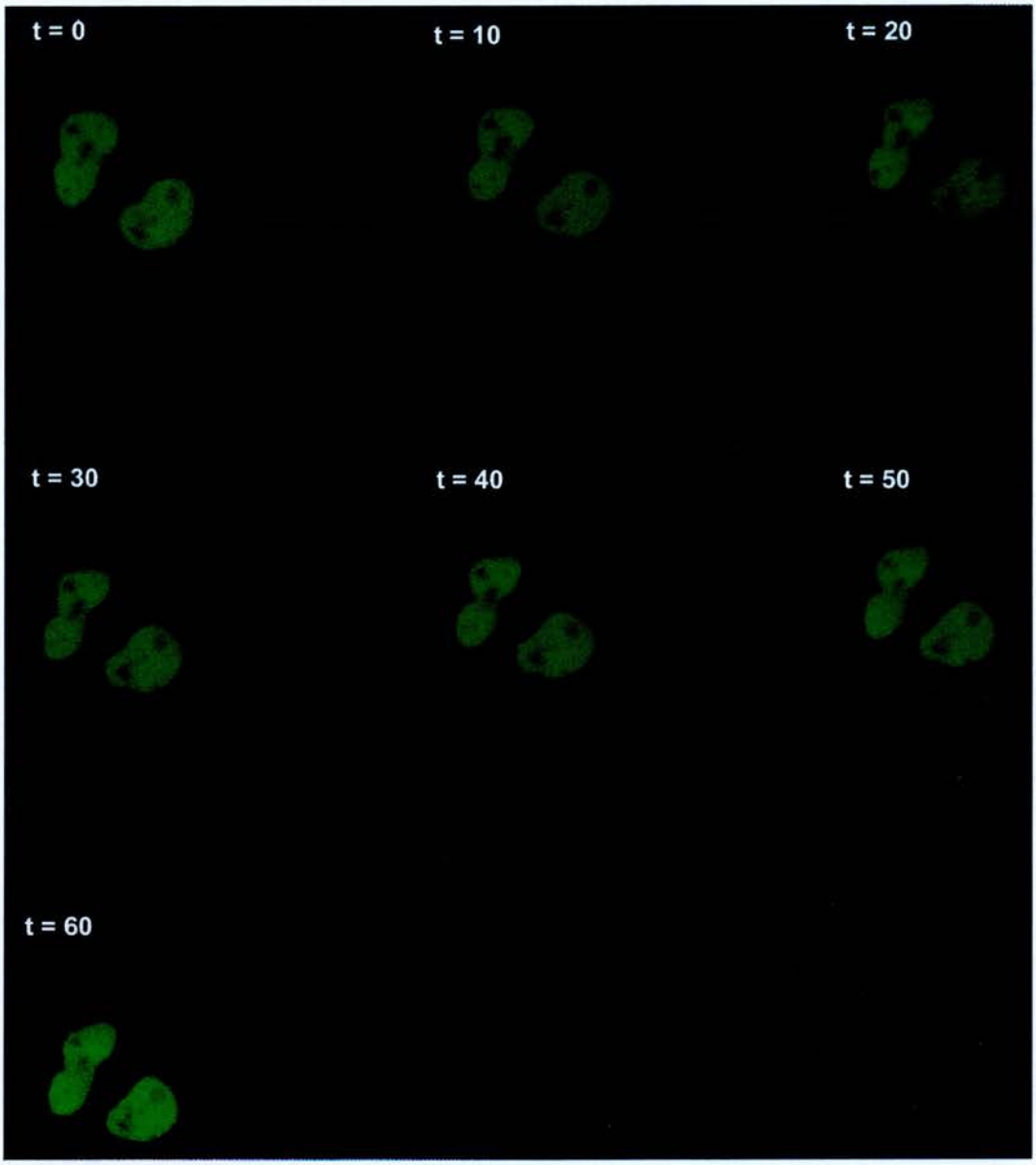


Figure 6.3a. ERβ2-EGFP expression in Hep G2 cells with addition of 10^{-8}M EGF. The ERβ2-EGFP construct transiently transfected into Hek 293 cells, maintained in culture for 48 hours and analysed under a confocal microscope. Cells were washed and treated with 10^{-8}M EGF. Confocal images taken over 60 minutes starting from time (t) 0, at 10 minute intervals.

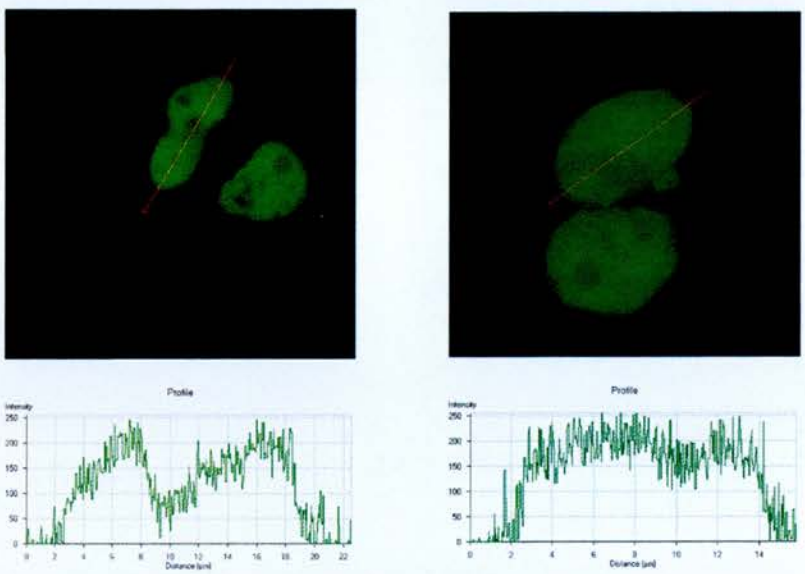


Figure 6.3b. ERβ2-EGFP expression in Hep G2 cells with addition of 10^{-8}M EGF for 60 minutes. Images are of two cell nuclei showing a graph of the intensity profile of the fluorescent ER. The graph demonstrates the intensity of the fluorescence (y axis) along the length of the line drawn through the nucleus, in the direction of the arrow (x axis). All graphs shown have the same y axis values.

6.3.2 Detection of EGFr and IGFr mRNAs in Cell Lines

Primers specific for EGF and IGF receptors were used to perform PCR analysis on pools of cDNA prepared from different cell lines (*Figure 6.4*). Following PCR amplification products of the expected sizes were observed on gels: EGFr gave a band at 237 bp, IGFr at 166 bp and GAPDH at 280 bp. EGFr was detected in Hek 293, Ishikawa, LnCap and Hela cells, but was absent from Hep G2 and Cos 7 cells (*Figure 6.4*; top panel). IGFr mRNA expression was detected Hek 293, Hep G2, Ishikawa, LnCap and Hela cell lines, but was absent from Cos 7 cells (*Figure 6.4*; middle panel). The positive control, GAPDH, was detected in all cell lines but was absent in the negative control.

6.3.3 EGFr Expression in Cell Lines Identified by Immunocytochemistry

Immunocytochemical evaluation of the Hek 293 and Hep G2 cell lines confirmed RT-PCR results. There was no detectable EGF receptor protein staining in Hep G2 cells, but immunopositive staining was present in some Hek 293 cells (*Figure 6.5*) within the cytoplamsic and membrane regions.

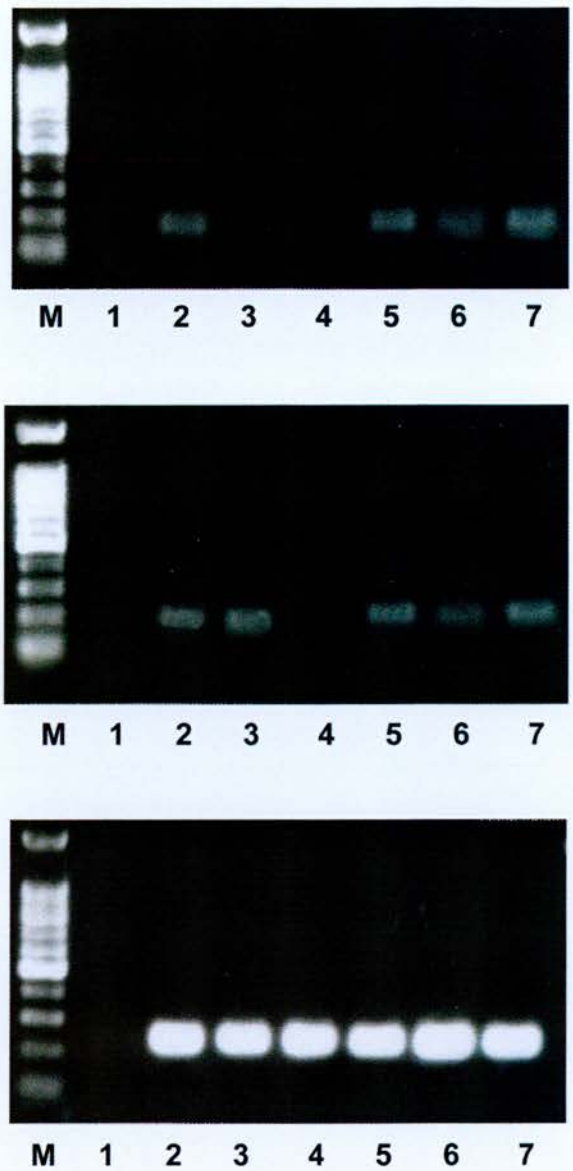


Figure 6.4. Detection of EGF receptor and IGF receptor mRNAs in cDNAs pools prepared from cell lines. RT-PCR was performed using specific primers to detect EGFr (top panel; 237 bp) and IGFr (middle panel; 166 bp) and GAPDH (lower panel; 280bp) as a positive control. The cell lines tested were (1) blank, (2) Hek 293, (3) Hep G2, (4) Cos 7, (5) Ishikawa, (6) LnCap, (7) Hela. 100pb marker was run on all gels.

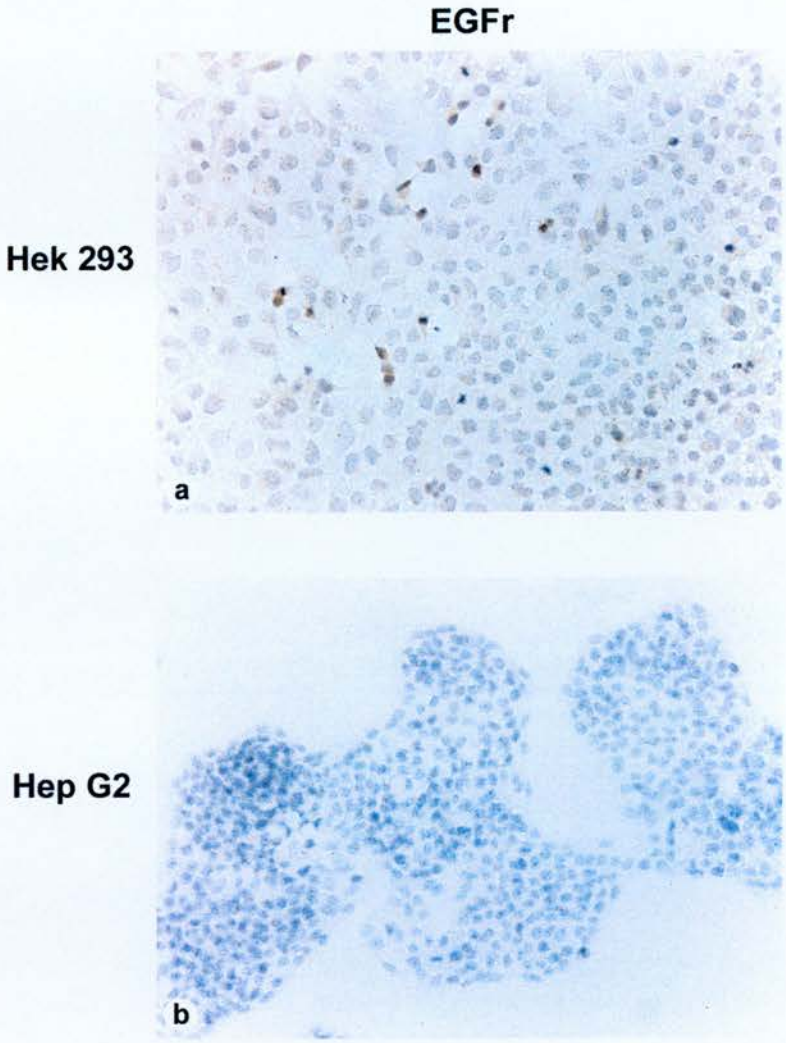


Figure 6.5. Immunocytochemical analysis of EGFr protein expression in Hek 293 and Hep G2 cell lines. Hek 293 cells (a) and Hep G2 (b) were stained with specific EGFr antibodies. In some Hek 293 cells the cytoplasmic and plasma membrane regions stained immunopositive for the EGFr protein, Hep G2 cells did not stain immunopositive for the EGFr protein. Magnification is x20.

6.3.4 EGF Transient Transfections

In transient transfection studies using Hek 293 cells, human ERα, ERβ1 and ERβ2 were stimulated to activate gene transcription via a 3x ERE-Luc reporter, in the presence of EGF (*Figure 6.6*). A dose response increase in ERE activation was observed for ERα, ERβ1 and ERβ2 with 10^{-9}M and 10^{-8}M EGF, however at higher concentrations (10^{-7}M to 10^{-5}M) activation was reduced and showed no increase in activation compared to the control. ERα showed a slightly greater maximum fold increase (reaching 5.7-fold) whereas ERβ1 (4.2-fold) and ERβ2 (4.1-fold) showed a similar extent of activation. In Hep G2 cells, EGF did not activate ERα, ERβ1 nor ERβ2 (*Figure 6.7*).

6.3.5 IGF Transient Transfections

RT-PCR has detected IGF receptor mRNA within Hek 293 and Hep G2 cell cDNA pools, therefore transient transfections in Hep G2 cells with ERα, ERβ1 and ERβ2 and a 3x ERE-Luc reporter were performed with IGF treatment. These transfections have demonstrated that IGF has the potential to activate gene transcription through ERα (*Figure 6.8*). A maximal activation in gene transcription by the ERE reporter was observed with 10^{-7}M IGF (4.5-fold increase). However, no increase in gene transcription was observed with ERβ1 or ERβ2 constructs.

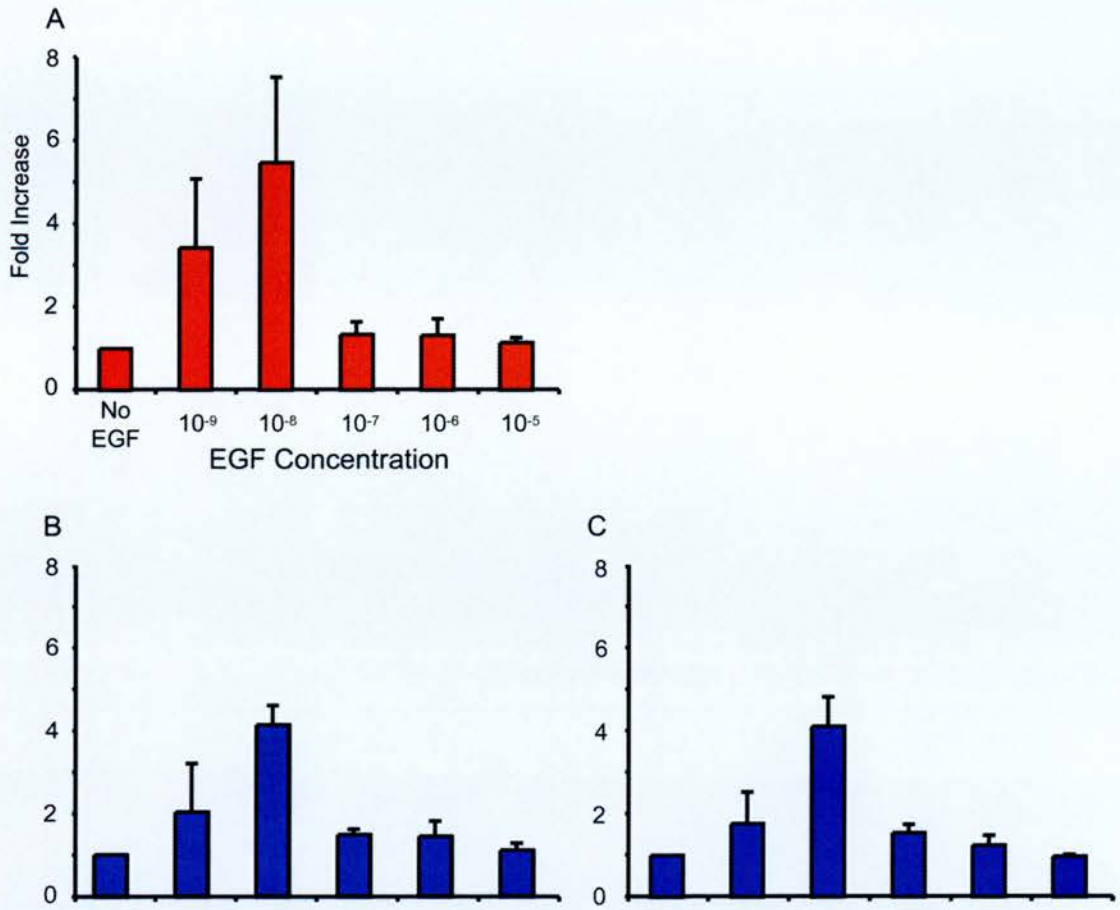


Figure 6.6. EGF dose response with ER transfected Hek 293 cells. Hek 293 cells were transiently transfected using JetPEI transfection reagent, with hERα (A), hERβ1 (B) or hERβ2 (C), the 3x ERE-Luc reporter and the pRL-CMV internal control. Cells were treated with a vehicle, or increasing concentrations of EGF, ranging from 10^{-9}M to 10^{-5}M. Data are displayed as fold induction of luciferase activity over the un-stimulated cell. Data are the mean +/- SEM of 3 experiments.

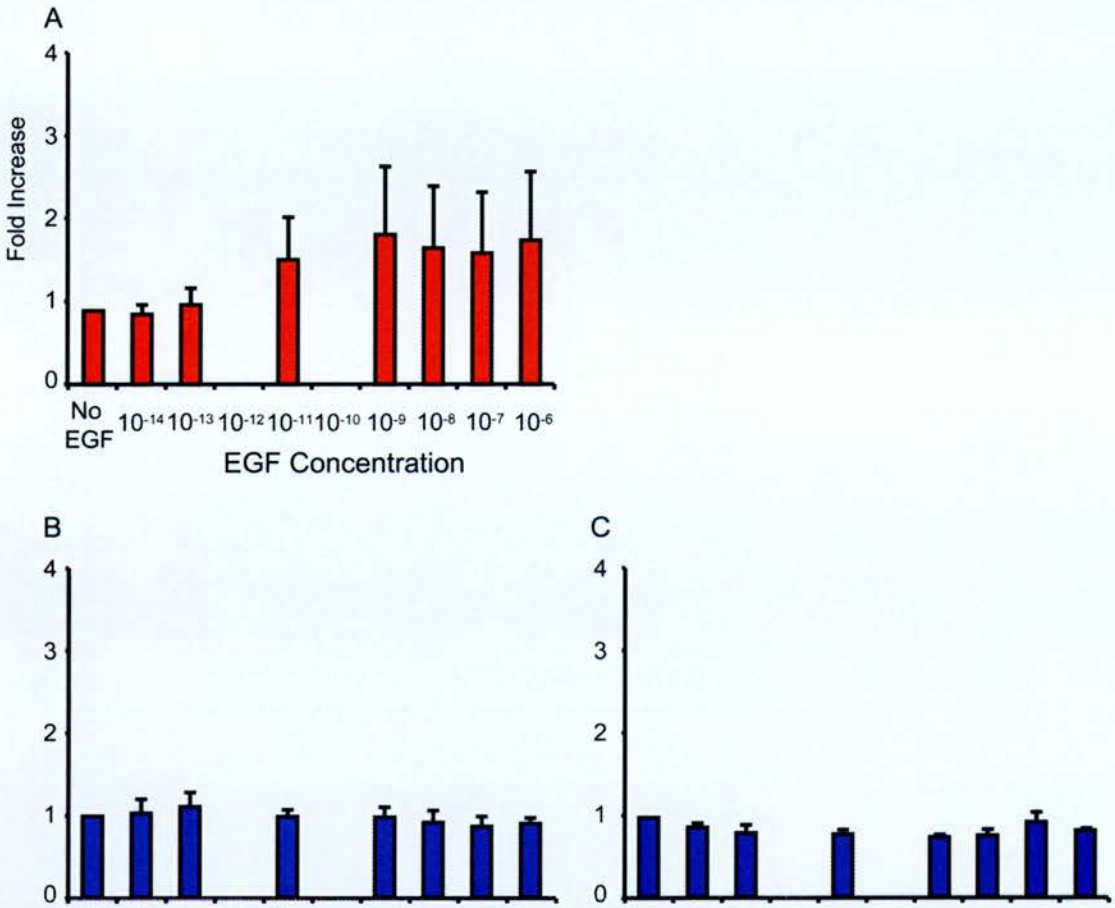


Figure 6.7. EGF dose response with ER transfected Hep G2 cells. Hep G2 cells were transiently transfected using JetPEI transfection reagent, with hERα (A), hERβ1 (B) or hERβ2 (C), the 3x ERE-Luc reporter and the pRL-CMV internal control. Cells were treated with a vehicle, or increasing concentrations of EGF, ranging from 10^{-14}M to 10^{-6}M. Data are displayed as fold induction of luciferase activity over the un-induced cell. Data are the mean +/- SEM of 3 experiments.

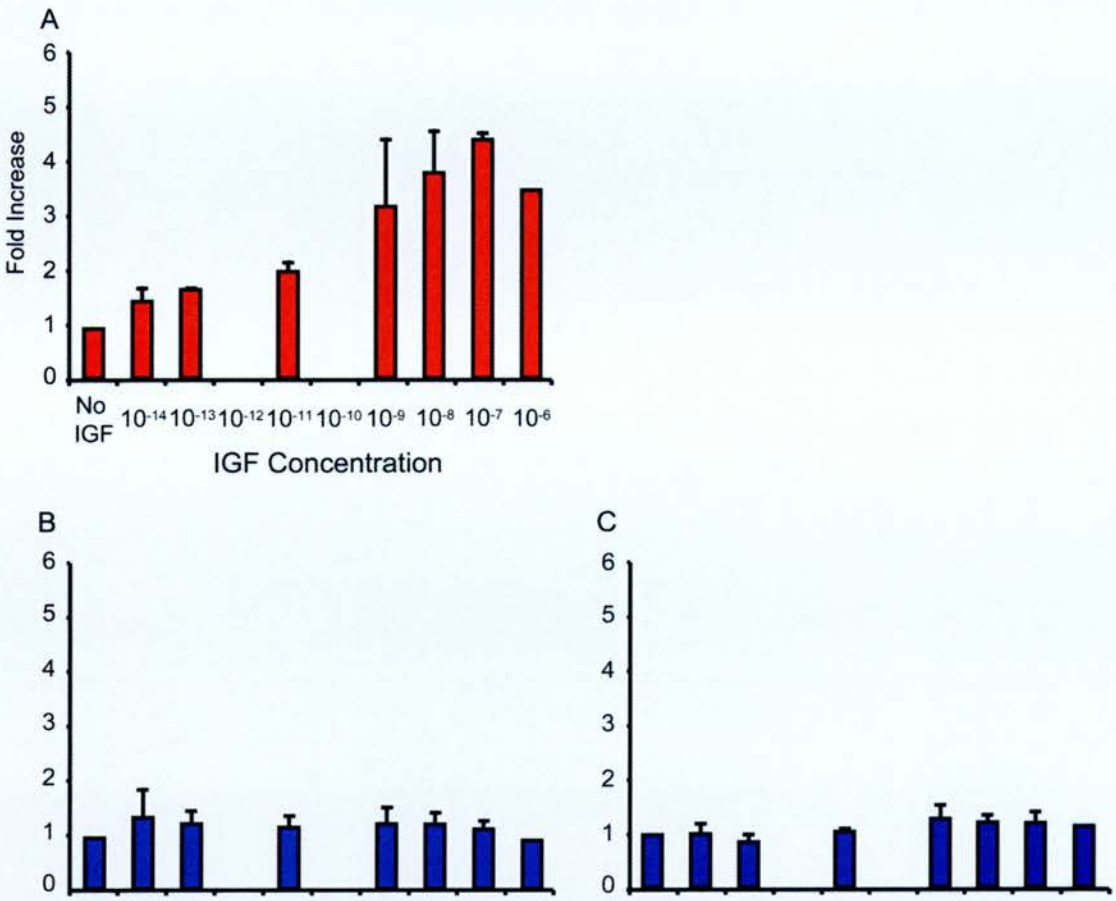


Figure 6.8. IGF dose response with ER transfected Hek 293 cells. Hek 293 cells were transiently transfected using Gene Porter transfection reagent, with hERα (A), hERβ1 (B) or hERβ2 (C), the 3x ERE-Luc reporter and the pRL-CMV internal control. Cells were treated with a vehicle, or increasing concentrations of IGF, ranging from 10^{-14}M to 10^{-6}M. Data are displayed as fold induction of luciferase activity over the un-induced cell. Data are the mean +/- SEM of 3 experiments.

6.3.6 p44/p42 MAP Kinase Detection

Hek 293 and Hep G2 cells were incubated for 48 hours in media containing either 10% charcoal stripped fetal calf serum or 10% serum replacement and then analysed for their MAP kinase content (*Figure 6.9*). A nuclear protein extract was prepared from the cells. On Western blots Hek 293 and Hep G2 cells both showed strong bands for p44 and p42 MAP kinases in extracts from cells incubated in both serum or serum free extracts. The phosphorylated forms of MAP kinases are also present in both cell lines, although the bands are stronger for the extracts incubated with the serum replacement. The bands shown on the Western correspond to 44 kDa and 42 kDa along with the positive control for the two different MAP kinase antibodies.

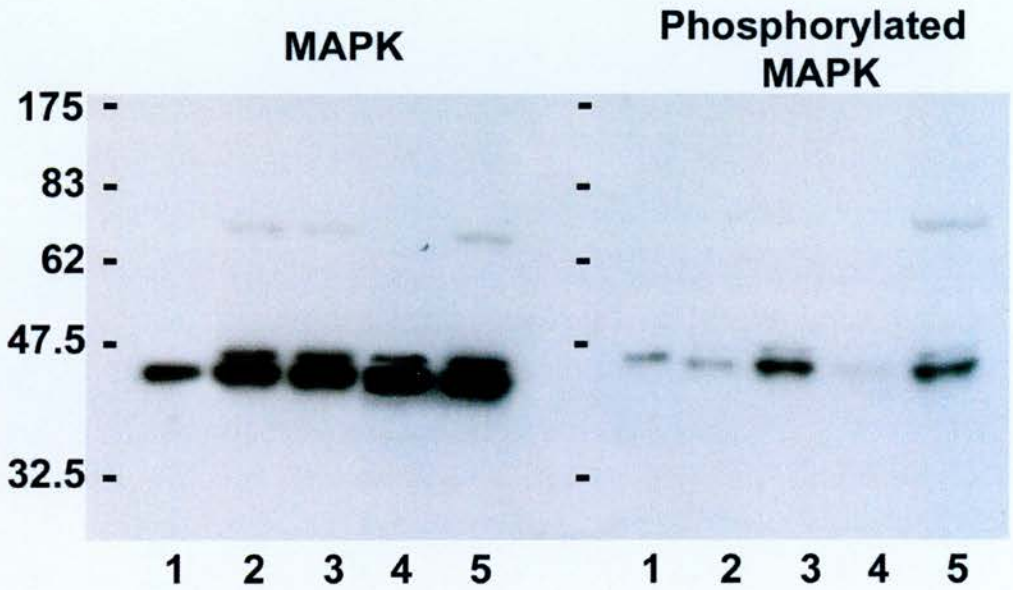


Figure 6.9. Western analysis for the presence of MAPK and Phosphorylated MAPK from the extracts obtained from Hek 293 and Hep G2 cells. Samples tested with the specific antibodies were (1) positive control, (2) Hek 293 cells treated with fetal calf serum, (3) Hek 293 cells treated with serum free media, (4) Hep G2 cells treated with fetal calf serum, (5) Hep G2 cells treated with serum free media. SDS Protein markers were run on the gel.

6.3.7 EGFr Expression in Testes Identified by Immunohistochemistry

Immunohistochemical evaluation of human, macaque and marmoset testes (*Figure 6.9*) detected immunopositive staining for EGF receptor protein in spermatogonial germ cells for all three species. In the human testis few EGFr positive cells were detected reflecting both the variety of spermatogonia and also poor tissue preservation. The receptor protein was localised to the cytoplasm of the germ cells and around the membrane, consistent with the previously published data. Germ cells were easier to identify in fixed sections of macaque and marmoset testes (*Figure 6.9.b, c*).

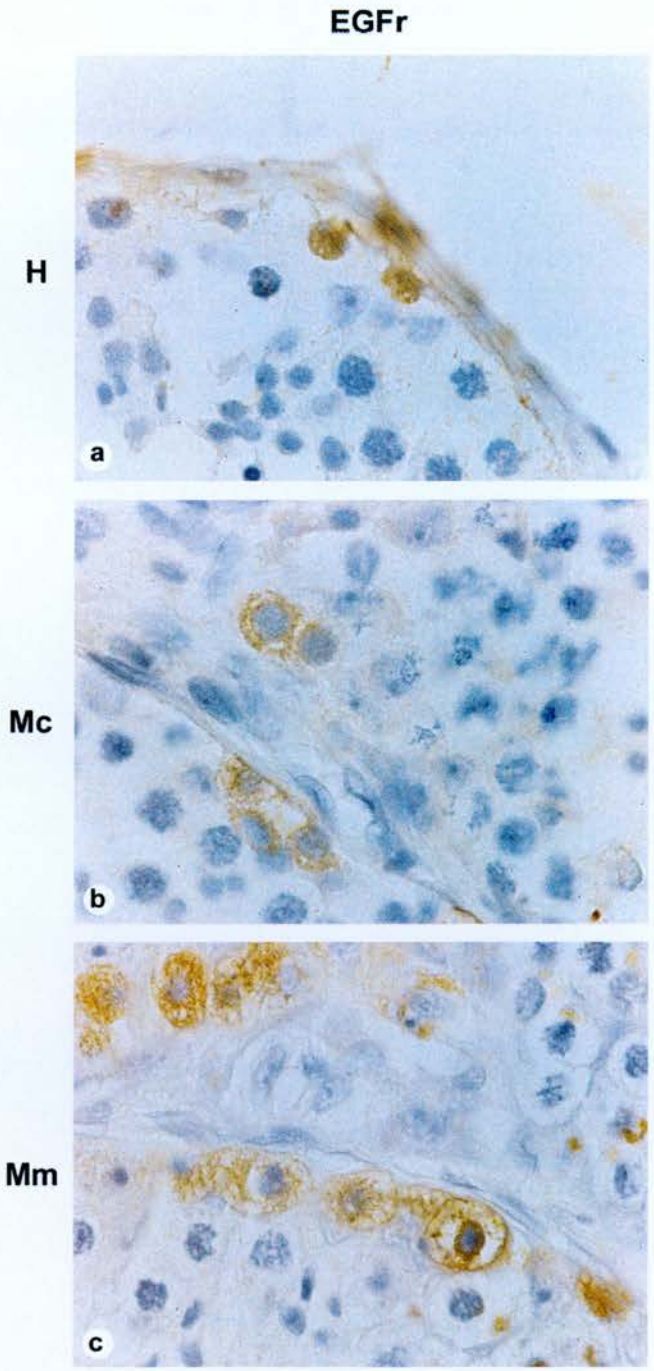


Figure 6.10. Immunohistochemical analysis of EGF receptor protein expression in the testis of the human, macaque and marmoset. Sections from human (a), macaque (b), and marmoset (c) were stained with the EGFr specific monoclonal antibody. Immunopositive staining was observed in the cytoplasm and cell membrane of germ cells in all three species. Unspecific staining, possibly of the acrosome, was observed in the marmoset section. Magnification is x 100.

6.4 Discussion

In the present study it has been demonstrated that cells which express the growth factor receptor can activate transfected ER constructs to induce gene transcription from an ERE promoter in the presence of growth factors probably via the MAP kinase signalling pathway. Confocal analysis of Hek 293 cells transfected with FP-tagged ERβ2 variant protein has shown that this "oestrogen" receptor can redistribute from a diffuse distribution to a discrete focal pattern within 10 minutes in the presence of EGF. However, in Hep G2 cells this activation of ERβ2 was not observed. RT-PCR analysis revealed that EGFr mRNA was present in Hek 293 cells but absent in Hep G2 cells, consistent with EGF stimulation of a phosphorylation cascade resulting in activation of the ER only being detectable in the Hek 293 cells. The activation of the ERE-Luc reporter by the ERβ2 in EGF treated cells was in marked contrast to the lack of transcriptional response in the E_2 treated cells.

The ERβ2 variant is truncated at the C-terminus and therefore lacks vital amino acids encoding for the AF-2 region within the LBD which is required to activate gene transcription. However, if there is an alternative pathway such as a growth factor induced MAP kinase signalling cascade which has the ability to phosphorylate the ERβ2 and induce gene transcription, it may explain a possible function of the ERβ2 variant. To assess whether cells which endogenously express the EGF receptor can activate an ERE-Luc reporter gene via this alternative pathway, transient transfections were performed. Both Hek 293 cells, which express EGFr, and Hep G2 cells which don't, were used in transient transfection studies. The ERα, ERβ1 and ERβ2 all initiated gene transcription of the ERE-Luc reporter with low (10^{-9}M and 10^{-8}M) concentrations of EGF in Hek 293 cells. As expected due to the absence of EGRr, the ER constructs transfected into the Hep G2 cells were unable to initiate transcription. However, IGF mRNA was found to be present in both Hek 293 and Hep G2 cells by RT-PCR, therefore transient transfections were performed to assess whether IGF can activate gene transcription through the ER. In Hep G2 cells only ERα was activated to stimulate the ERE-Luc reporter with IGF, in a dose responsive manner.

The 3x ERE-Luc reporter gene was stimulated to activate gene transcription via the ERα, ERβ1 and ERβ2 at a low level compared to when ERα and ERβ1 are activated by E_2. This minimal induction in reporter activity has been described in a

recent publication; Marquez *et al* used hERα and an ERE reporter in Cos 7 cells and reported that transcriptional activity was increased by 2-fold compared to E_2 stimulation which resulted in a 14-fold increase in transcription (Marquez *et al.*, 2001). This published increase in activation corresponds to the 4-fold to 6-fold increase I have demonstrated with ERα, ERβ1 and ERβ2 in Hek 293 cells, compared to the 30-fold increase in activity with ERα in the presence of E_2.

My results have demonstrated that although there are detectable levels of EGF receptor mRNA and IGF receptor mRNA in Hek 293 cells, the protein expression within these cells is low. Therefore, the low levels of transcriptional activation of the oestrogen receptors in the presence of EGF or IGF may be due to the lack of EGF or IGF receptors within the membrane and cytoplasm of the cells transiently transfected with the ER construct.

Human ERα contains a Serine residue at position 118 within the AF-1 region, which is phosphorylated by MAP kinase signalling cascades (Marquez *et al.*, 2001 and reviewed by Rochette-Egly, 2003). Tremblay *et al* have analysed the mouse ERβ1 and showed it also contains a phosphorylation site (Ser124). Sequence alignment of the human ERα, ERβ1 wild type and ERβ2 variant peptides (Chapter 3) demonstrates a Serine118 residue in ERα and two conserved Serine residues in both ERβ1 and ERβ2 (positions 87 and 106). These residues in the ERβ1 have been documented previously to be phosphorylated by the MAP kinase signalling cascade (Rochette-Egly, 2003) but no previous data has shown these residues to be present in hERβ2. Therefore both hERβ1 and hERβ2 are able to be phoshorylated in the same manner, and as ERβ2 contains the highly conserved DBD and dimerisation regions that ERβ1 contains (Ogawa *et al.*, 1998c) it could be functionally activated.

It has previously been reported that IGF induces phosphorylation differently to EGF, as shown with ERα (Flint *et al.*, 2002). Therefore it may be that IGF can activate ERα, but not ERβ1 or ERβ2, through the phosphorylation of Ser118 or through one of the other serine residues (104, 105, 167) that are not present in the AF-1 region of hERβ. Flint *et al* demonstrated that in the presence of IGF, phosphorylated MAP kinase p44 or p42 did not increase, in contrast to the increase observed with EGF, however they did note that there was a high endogenous level of p44 and p42 that could be "masking" any effect (Flint *et al.*, 2002).

Western analysis to detect phosphorylated and non-phosphorylated MAP kinases with the cells was performed. Both p44 and p42 MAP kinases (Erk1 and Erk2) function in the protein kinase cascade that plays a critical role in the regulation of cell growth and differentiation. MAP kinases are activated through a wide variety of extracellular signalling pathways including those activated by growth factors. Activation of MAP kinases and signalling cascades occurs through phosphorylation of threonine and tyrosine residues by upstream MAP kinase kinase (MEK) activities (reviewed in the literature review *Figure 1.17*) (Levin, 2003). One aim in this study was to determine whether the presence of EGF could upregulate the MAP kinase signalling pathway. However, unstimulated Hek 293 and Hep G2 showed a high endogenous level of phosphorylated MAP kinase activity (as shown in *Figure 6.9*) therefore it was not possible to determine whether the phosphorylated MAP kinase was upregulated upon EGF stimulation. The cells were originally maintained in 10% charcoal stripped fetal calf serum, but as these cells showed a high level of phosphorylated and non-phosphorylated MAP kinase, a serum replacement was used to try to reduce this, however endogenous MAP kinase activity remained at a high level. The phosphorylated MAP kinase showed less intense bands on the Western (*Figure 6.9*) but the positive control (supplied with the antibody) also showed a less intense band, therefore indicating that it was likely due to the antibody. Therefore, the Western results have demonstrated that the MAP kinase pathway is active within both the Hek 293 and the Hep G2 cells, so activation of ERs via an alternative growth factor pathway is viable. Flint *et al* have previously demonstrated that phosphorylated p44 and p42 MAP kinases are increased in a dose responsive manner by EGF (Flint *et al.*, 2002) in stromal and endometrial cells. They therefore was reported that p44 and p42 may be responsible for the ER phosphorylation and activation in the cells (Flint *et al.*, 2002).

If the oestrogen receptors are activated through an alternative steroid ligand independent pathway such as the growth factor pathway, as demonstrated in these results, the physiological significance needs to be addressed. Immunohistochemical analysis was performed on the testes of human, macaque and marmoset, where it has previously been demonstrated (Chapter 3 and Saunders *et al.*, 2002a) that both ERβ1 and ERβ2 are expressed. In the present experiments EGFr was detected in the cytoplasm and membrane of spermatogonial cells within the testes. Data from Chapter 3 and previous published data (Saunders *et al.*, 2002a) has shown that

ERβ2 is present in the germ cells of the human using specific anti-human ERβ2 antibodies, and that ERβ is present in these germ cells in human, macaque and marmoset (Saunders *et al.*, 2001). Therefore it is physiologically possible that EGF can activate the EGF receptor in the cell membrane, which in turn phosphorylates and hence activates the MAP kinase signalling cascade within the cytoplasm. This would result in the phosphorylation of ERβ1 wild type and ERβ2 variant in the nucleus and induction of the classical ERE to initiate gene transcription. In future studies it would be valuable to determine EGFr expression in tissues other than the testes and to perform dual immunohistochemical experiments so that both ERβ2 and EGFr protein expression could be observed in the same section and therefore be assured that both receptors are localised within the same cell. However, in this study both the ERβ2 and the EGFr antibodies were raised in mouse, therefore a different EGFr antibody needs to be optimised before dual experiments can be carried out. The co-expression studies of ERα, ERβ1 and ERβ2 and the EGF receptor is required in all tissues which have been reported to express the oestrogen receptors.

The importance of growth factor induction of ER transcriptional activity to normal physiological responses is not clear at the present time. It is possible that growth factors maintain moderate levels of ER transcriptional activity when oestrogen levels are low. Alternatively, signalling cascades initiated by growth factors binding to their receptors may modulate transcriptional activity of ligand-occupied ER and thereby increase the magnitude of target gene expression (Smith, 1998). Low oestrogen levels could increase growth factor expression and together these factors may promote a stronger or more sustained biological response in target tissue. It is also possible that cross-talk pathways may sensitise the ER to suboptimal stimulation by low levels of oestrogens and thereby promote biologically meaningful responses under conditions in which the ligand stimulus alone would be unable to generate a significant response (Smith, 1998).

Altered elements in growth factor signalling pathways, such as receptor over expression, may directly influence steroid hormone action in human breast cancers (Nicholson *et al.*, 1999). Enhanced cross-communication between growth factor receptor pathways and ER during cancer progression could contribute to ER activation in the absence of ligand. Current findings show that the EGF receptor

plays a leading role in the progression of breast tumours, therefore further delineation of these complex pathways in breast cancer cells may lead to the design of novel therapies that combine anti-growth factor signalling strategies with anti-hormone measures. Cross-communication between peptide growth factor pathways and ER may prove to be very important in modulating hormonal activity in normal and aberrant tissue.

Chapter 7

General Discussion

Oestrogens are key regulators in male and female reproductive function, as well as playing an important role in other tissues including the cardiovascular system and bone. Oestrogen action is mediated via high affinity nuclear receptors expressed in target tissues. Studies in this thesis were aimed at expanding our understanding of the impact of endogenous and exogenous oestrogenic ligands on cell function by focusing on expression and activation by oestrogen receptors.

In human as well as other mammals, two oestrogen receptor genes; ERα and ERβ, have been cloned. A number of splice variant isoforms of human ERβ have been described (Inoue *et al.*, 1996; Lu *et al.*, 1998; Moore *et al.*, 1998; Ogawa *et al.*, 1998c). These splice variants do not appear to exist in rodents and expression in other species has not been investigated. In this thesis I have concentrated on the ERβ wild type and the ERβ2 splice variant.

The aims of this thesis were several fold, to begin with it was necessary to determine whether ERβ wild type and ERβ2 variant forms exist in primates, as well as in human, and to determine the localisation of these receptors within reproductive tissues. This was performed using RT-PCR from tissue and cell cDNA pools and immunohistochemical and immunocytochemical analysis using anti-human ERβ1 and anti-human ERβ2 antibodies.

Once the expression was determined, fluorescently tagged ER constructs were used to examine the localisation and redistribution of the receptors in the absence and presence of different oestrogenic ligands using a confocal microscope. After observing the differences in the activation potential of oestrogenic ligands, functional studies were performed to assess the ability of these ligands to initiate gene transcription via ERα, ERβ1 or ERβ2 using ERE-luciferase reporter constructs.

Sequence analysis has revealed that the ERβ2 variant does not contain the AF-2 region and limited functional studies have suggested that it cannot be activated by addition of ligand. My results extended these findings and showed that FP-tagged hERβ2 did not redistribute upon addition of ligand and was unable to activate an ERE-Luc reporter in transient transfections studies. However immunohistochemical

investigations have detected ERβ2 protein in several tissues and therefore investigations into alternative activation pathways were performed to determine whether it could be activated in the absence of oestrogenic ligand.

7.1 Differential Expression of the Oestrogen Receptor beta in the Reproductive Tract

The results described in this thesis as well as recently published data, have demonstrated that there is differential expression of the ERα, ERβ1 and ERβ2 proteins in human tissues. ERβ1 and ERβ2 mRNAs were detected in human and primate tissues by RT-PCR, however immunohistochemical analysis failed to detect ERβ2 protein in macaque or marmoset tissue. In this thesis the anti-hERβ2 antibody had been raised to a peptide equivalent to the C-terminus of human ERβ2. Sequence alignment of the primate ERβ2 proteins, predicted from analysis of the open reading frames, revealed that in the macaque the ERβ2 protein was truncated due to an insertion of a short intronic sequence which introduced a premature stop codon. Although the marmoset ERβ2 protein was similar in size to that found in human, the sequence found at the C-terminus was not conserved.

In the human and primate ovary ERα and ERβ are differentially expressed within the granulosa cells of the developing follicles (Saunders *et al.*, 2000). From my results it was observed that ERβ2 protein is expressed within the ovary of the human in a similar distribution to that of ERβ1, whilst RT-PCR has detected ERβ1 and ERβ2 mRNA in ovary cDNA pools from human, macaque and marmoset consistent with previous studies (Henderson *et al.*, 2003; Saunders *et al.*, 2000).

It has previously been demonstrated that in the endometrium ERα and ERβ are co-expressed in multiple cell types including the stromal and epithelial cells lining the glands, but only the ERβ protein is present in the endothelial cells lining walls of blood vessels (Critchley *et al.*, 2001). During the proliferative phase ERα mRNA is 300-fold that of ERβ mRNA (Matsuzaki *et al.*, 1999). Henderson *et al* have shown that both ERβ1 and ERβ2 mRNAs are expressed in the endometrium (Henderson *et al.*, 2003). In the present studies ERβ1 and ERβ2 mRNAs were detected in human, macaque and marmoset endometrial cDNA pools and pattern of ERβ1 protein expression was similar in macaque, marmoset and human endometrial tissues

sections. Consistent with other studies from our laboratory (Henderson *et al.*, 2003) ERβ2 protein was shown to be immunolocalised to human endometrium.

The immunohistochemical results from my studies have demonstrated for the first time that primate placental tissues contain ERβ1 protein, expressed within the nuclei of perivascular cells surrounding the blood vessels and the syncyiotrophoblast. The same pattern of expression was also observed in human placental tissue. However, immunohistochemical analysis and RT-PCR failed to detect ERβ2 protein or mRNA in the human, macaque or marmoset placental tissues.

In human and primate testes, in contrast to rodents, ERα mRNA and protein are not expressed (Pais *et al.*, 2003; Saunders *et al.*, 2001). In the current study RT-PCR has detected mRNA for both ERβ1 and ERβ2 in human, macaque and marmoset testes cDNA pools. ERβ1 protein has been shown in this study and in previous studies (Saunders *et al.*, 2002a) to be present in the nuclei of multiple cell types including pachytene spermatocytes and round spermatids. Immunohistochemical analysis showed staining was less intense in Sertoli cells than in the germ cells in human and primates. The most intense immunopositive staining for ERβ2 protein in human testes was detected in Sertoli cells and spermatogonia, with low or variable levels of expression in some spermatocytes.

The results from this thesis indicate that the marmoset is a good model in which to examine the impact of oestrogens on the reproductive organs as it expresses similar oestrogen receptors to the human and in a similar distribution. The human and marmoset ERβ1 and ERβ2 peptide sequences are highly conserved, except for the last five amino acids in the C-terminal region of the marmoset ERβ2.

Within reproductive tissues the expression of ERα and ERβ protein is not constant especially in abnormal tissue. For example ERβ2 expression is upregulated whereas ERβ wild type expression is down regulated in breast cancer tissues, therefore suggesting that ERβ1 may have a role in normal breast tissue oestrogen signalling but in breast cancer, expression of ERβ2 increases (Omoto *et al.*, 2002). Saji *et al* reported that ERβ2 was expressed in breast cancer tissue, whereas ERβ1 was absent (Saji *et al.*, 2002). However, Saunders *et al* found no quantitative relationship between staining for the two ERβ subtypes, although they did show both receptors were present (Saunders *et al.*, 2002b).

Fuqua *et al* have demonstrated that several ERβ variants are present in breast cancer cell lines (Fuqua *et al.*, 1999). A recent publication has reported that in normal and neoplastic breast tissues the level of expression of the C-terminally truncated ERβ variants; ERβ2 and ERβ5, is markedly higher than the full length ERβ1. These data suggest that the ERβ2 and ERβ5 variants may have a role in modulating oestrogen and possibly anti-oestrogen action in human breast cells (Peng *et al.*, 2003).

Another example of a tumour tissue in which expression of ERβ1 may modulate oestrogen responsiveness is prostate cancer. There have been many conflicting reports of ERα or ERβ expression being upregulated in cancer progression (Pasquali *et al.*, 2001; Royuela *et al.*, 2001; Tsurusaki *et al.*, 2003). Overall the results demonstrate that both ERα and ERβ are present within the prostate and that oestrogens exert their effects on human prostate through ERβ1 (Pasquali *et al.*, 2001). In benign prostatic hyperplasia and prostate cancer both ERα and ERβ are upregulated, but ERβ to a greater extent (Royuela *et al.*, 2001).

Anti-oestrogens, for example, tamoxifen, are agonists when signalling through the AP-1 element with ERβ and therefore over expression of ERβ1 in tumours may explain a lack of effectiveness of anti-oestrogen therapy in some cancer patents (Speirs *et al.*, 1999). Hence, in cancers such as breast cancer appropriate ERβ-specific ligands, perhaps in combination with ERα-specific ligands may be useful in improving therapy.

7.2 Functional Analysis of the Oestrogen Receptors

The localisation studies proved an important initial step in determining the sites of oestrogen receptor expression and an indication of cell types that oestrogenic ligands could be expected to target. Subsequent studies focused on the ER isoforms and their ability to activate gene transcription with various oestrogenic ligands. In addition to transient transfections of individual receptors, transcriptional activity of co-transfected ERs (ERα/ERβ1, ERα/ERβ2, ERβ1/ERβ2) was assessed as this would reflect the ER expression in cells within the reproductive tissues. To do this two experimental strategies were employed, firstly observation studies using FP-tagged ERs and secondly, assessment of transcriptional activity using ERE-luciferase reporter constructs.

In the absence of ligand the ERs tagged to the fluorescent proteins were diffusely distributed within the cell nucleus. Upon addition of oestrogenic ligands receptors had the capacity to redistribute into discrete foci as demonstrated in previous studies (Htun *et al.*, 1999; Stenoien *et al.*, 2001a). Transient transfections with an ERE-luciferease reporter were also performed to ascertain whether the ER redistribution to focal regions was consistent with an increase in reporter gene activation.

Ligand-activated oestrogen receptors bind with high affinity to specific DNA sequences, known as oestrogen response elements (EREs), located within the regulatory regions of target genes. The sequence of the EREs are reported to influence receptor activity (Klinge *et al.*, 1997). It has been shown that if the ERE sequence deviates from the consensus sequence by a single base pair, the receptor exhibits a reduced affinity and decreased transcriptional activity (Wood *et al.*, 2001). Similar results were observed in the present study and additionally the cell types used for transfections were also found to influence the results obtained. For example, the single oxytocin ERE (OT) which has one base change compared to the consensus vitellogenin (Vit) ERE, activated gene transcription minimally with ERα and ERβ1 in Hep G2 cells, although in Hek 293 cells ERα was able to activate the OT ERE to a greater extent than a single consensus ERE. Highest levels of reporter gene activation were observed with the construct containing three tandem copies of the ERE consensus sequence. This high level of gene transcription is consistent with previous published data (Klinge, 1999; Tyulmenkov *et al.*, 2000) and was observed in both cell lines using either ERα or ERβ1. This construct was used throughout the rest of the studies described in the thesis.

It is well documented that different oestrogenic ligands have the ability to activate ERα and ERβ1 to different extents and this has been attributed by their differential binding affinity, ability to alter the ER conformation and thereby influence co-factor recruitment (Barkhem *et al.*, 1998; Kuiper *et al.*, 1997). In this study I analysed the redistribution and transcriptional activation of the ERs in response to different concentrations of the natural oestrogenic ligands 17β-oestradiol, 3βAdiol and genistein. The recently developed synthetic ligands PPT™ and DPN™ which are specific to ERα and ERβ1 respectively, were also used. The results demonstrated

that a redistribution of FP-ERs upon addition of ligand was consistent with an increase in ERE-Luc reporter gene activation.

However, differences in the subcellular distribution pattern of the FP-ERs were observed. For example, following exposure to ligand FP-ERβ1 tended to form discrete foci whereas more diffuse foci were observed using FP-ERα. Addition of different oestrogenic ligands also resulted in variations in the time it took for the ER to redistribute. The functional studies correspond to these differences and have also demonstrated that the ligands have a dose dependent impact on activation of hERα as well as human, macaque and marmoset ERβ1. As expected the human FP-ERβ2 which lacks the AF-2 region, did not redistribute following exposure to ligand nor did it activate gene transcription.

In summary, E_2 initiated redistribution of the FP-ER most rapidly and initiated the greatest transcriptional activation of the three natural ligands tested. Human ERα stimulated ERE reporter gene activation to a greater extent that hERβ1 in the presence of E_2 and was shown to redistribute more rapidly.

The DHT metabolite, 3βAdiol was found to activate ERE reporter gene transcription greatest with ERβ1 compared to ERα, in a dose response manner. Both ERs were observed to redistribute within the nucleus in Hek 293 and Hep G2 cells. However, ERα transfected into Hep G2 cells and stimulated with 10^{-7}M or 10^{-6}M 3βAdiol activated ERE reporter gene activation dramatically, possibly due to over stimulation of the ERα.

Exposure to 10^{-8}M of the ERβ-specific phytoestrogen genistein resulted in a slower redistribution of FP-ERα compared to FP-ERβ1. In addition, at higher concentrations of genistein (10^{-8}M to 10^{-6}M) ERβ1 stimulated ERE reporter gene activation to a greater extent than did ERα. These findings are consistent with previously publications that report genistein is an ERβ1-specific ligand with a much higher binding affinity for ERβ than ERα, although it exhibits only partial agonism with ERβ1 but full agonism with ERα (Pike *et al.*, 1999).

In line with previous data (Kraichely *et al.*, 2000) the synthetic ligand PPT™ activated ERα but not human ERβ1. However both macaque and marmoset ERβ1 were stimulated to activate reporter gene transcription by PPT™. This result has not

been previously published and I propose the reason why the primate ERβ1 proteins can be activated by PPT™ may be due to slightly different conformational changes in their LBD and AF-2 regions upon ligand binding compared to human ERβ1.

The ERβ-selective ligand, DPN™, incubated with FP-ERβ1 resulted in the redistribution of the ER into discrete foci, however unexpectedly FP-ERα redistributed into focal regions more rapidly. Functional assays were in agreement with the FP data and both ERs activated reporter gene transcription. Additionally, at high DPN™ concentrations (10^{-6}M), fold activation of ERE-Luc reporter was greater with ERα than ERβ1, whereas at low concentrations (10^{-7}M) this was the other way round and corresponds to previous publications (Meyers *et al.*, 2001; Ramsey *et al.*, 2003).

In summary the functional assays have both demonstrated that activiation of ERα and ERβ is influenced by the structure of the ligand as well as its concentration. The results have also demonstrated for the first time that macaque and marmoset ERβ1 are activated in a similar manner to human ERβ1, except with the novel ERα-specific ligand PPT™.

There is a growing body of evidence that ERα and ERβ expression may be differential or overlapping (Dotzlaw *et al.*, 1997; Saji *et al.*, 2000; Saunders *et al.*, 2002a; Saunders *et al.*, 2001). Depending upon whether one or both of the receptors are present in a cell, it is possible that homodimers (ERα/ERα or ERβ/ERβ) or heterodimers (ERα/ERβ) may be formed.

In Chapter 4 results were presented which showed that following exposure to E_2, FP-tagged hERα was present in the same foci as FP-hERβ1 or FP-hERβ2. Furthermore a similar pattern was seen when FP-hERβ1 and FP-hERβ2 were co-expressed. However, differences were noted in the behaviour of the receptors in Hek 293 and the Hep G2 cells. In Hek 293 cells ERα and ERβ1 co-localised in foci and ERE reporter gene activation was increased. ERα and ERβ2 did not show a clear focal pattern and it was difficult to observe foci occurring together, functional analysis showed a decrease in ERα's ability to activate the ERE reporter gene but there were variations between experiments. In contrast, in the same cell type FP-tagged hERβ1 and hERβ2 appeared to form heterodimers and surprisingly an increase in ERE reporter gene transcriptional activation was detected.

In Hep G2 cells FP-hERα and FP-hERβ1 were detected in the same foci and the ERE-Luc reporter gene expression was reduced compared with that observed with ERα alone. Co-transfections of FP-hERα and FP-hERβ2 resulted in detection of both proteins in some foci and a decrease in transcriptional activation of the reporter was also observed. The FP-tagged ERβ1 and ERβ2 were also able to redistribute together and similar to Hek 293 cells, the presence of ERβ2 resulted in an increase in gene transcription compared to that seen with ERβ1 alone.

As demonstrated in this thesis, the hERβ2 construct was unable to stimulate ERE-Luc reporter gene activity in the presence of steroids and FP-ERβ2 did not redistribute upon addition of ligand. However, in co-transfection studies FP-ERβ2 was redistributed into foci with ERα or ERβ1 and appeared to have an impact on reporter gene activation. I propose that the focal redistribution of ERβ2 observed when the protein is co-expressed with ERα or ERβ1 reflects heterodimerisation with the full length receptors and that after these bind ligand they "pull" the ERβ2 into focal sites and/or bind as a heterodimer to EREs.

Previously gel shift analysis has demonstrated in an artificial *in vitro* context, that ERα, ERβ1 and ERβ2 all have the ability to form homo- and heterodimers (Cowley *et al.*, 1997; Moore *et al.*, 1998). Transient co-transfections have been performed using hERα/ERβ1, hERα/ERβ2 and hERβ1/ERβ2 (Hall and McDonnell, 1999; Ogawa *et al.*, 1998c; Peng *et al.*, 2003). All of these studies have reported that co-expression of ERβ has an impact on ERα activated reporter gene expression. Ogawa *et al* and Peng *et al* showed a decrease in ERα induced reporter activation with increasing concentrations of ERβ1 (1:10 and 1:9 ratios respectively) using a 1x ERE reporter (Ogawa *et al.*, 1998a; Peng *et al.*, 2003). Whereas Hall *et al* demonstrated that there was no effect at high (10^{-7}M) ligand concentrations, but at lower concentrations (10^{-10}M) ERβ decreased ERα's activity when transfected at a 1:1 ratio with a 3x ERE reporter (Hall and McDonnell, 1999). Co-transfection of ERβ2 has been shown to decrease ERα's activity (Ogawa *et al.*, 1998c; Peng *et al.*, 2003). In co-transfections with ERβ1 and ERβ2 it has previously been demonstrated that the transcriptional activation remains the same as that seen with ERβ1 alone (Ogawa *et al.*, 1998c).

Direct comparisons between my studies and those previously published are difficult because different cell lines, different EREs and varying ER:ER ratios have been used. Overall I have demonstrated that ERβ2 acts to decrease ERα's activity via a 3x ERE-Luc reporter which is similar to previous publications, but acts to increase ERβ1's activity which has not been previously demonstrated.

Gel shift assays have shown that the ERα/ERβ1 heterodimer is capable of binding to the DNA with a similar affinity to that of the ERα homodimer (Ogawa *et al.*, 1998c) and the dimers were able to bind the co-activator SRC-1 to a similar extent in gel shift assays (Cowley *et al.*, 1997). However as the heterodimer has less transcriptional activity than the ERα homodimer it has been suggested that it may be less efficient at recruiting co-activators in cells (Peng *et al.*, 2003). In contrast to ERβ1, ERβ2 was demonstrated to be incapable of interacting with the co-activator TIF1α due to its lack of AF-2 region which is involved in co-factor recruitment, therefore suggesting that the lack of transcriptional activity with ERβ2 heterodimers may be partially due to ERβ2's inability to bind co-activators (Ogawa *et al.*, 1998c). Although it has been postulated that the ability to bind co-activators is not directly associated with the ability of the ERβ1 and ERβ2 variants to inhibit ERα activity (Peng *et al.*, 2003). The ERβ2 variant has a markedly reduced ability to bind to DNA (Moore *et al.*, 1998) and it is possible the ERα/ERβ2 heterodimers bind less well than the ERα/ERα homodimers to the ERE (Moore *et al.*, 1998; Ogawa *et al.*, 1998c). Therefore the reduced transcriptional activity may be due to a combination of factors including the reduced ability of the ERβ variants to form stable heterodimers and complexes at individual promoters compared with ERα homodimers.

If ERs are expressed together they form heterodimers, which under experimental conditions, are preferred over homodimers (Cowley *et al.*, 1997). Therefore, if it can be concluded that the presence of ERβ and the variants are able to modulate activity of ligand bound full length ERα or ERβ, it has physiological significance in conditions such as altered oestrogen sensitivity, for example in tumours as previously discussed.

The ER is not the only nuclear receptor with splice variants that are thought to possess a dominant negative ability. Alternative splicing of the human glucocorticoid

receptor (GR) generates a non-hormone binding receptor (hGRβ) that differs from the wild type receptor (hGRα) only at the C-terminal. It has been demonstrated that hGRβ inhibits the transcriptional activity of hGRα and has physiological consequences in patients with generalised and tissue-specific glucocorticoid resistence (Hecht *et al.*, 1997; Oakley *et al.*, 1999; Yudt and Cidlowski, 2001).

The steroid receptor co-activator (SRC-1) is one of a number of transcriptional modulators that are capable of potentiating the activity of ligand activated nuclear receptors including the oestrogen receptors (Klinge, 2001). The ER-co-activator interaction stabilises the formation of a transcription pre-initiation complex and facilitates the remodelling of chromatin at the target gene promoter (Leo and Chen, 2000).

There are two isoforms of the SRC-1; SRC-1e and SRC-1a, which diverge at their C-termini and are functionally distinct. The difference in activation between the two isoforms is thought to be due to a second activation domain that is CBP-independent and is suppressed in the SRC-1a isoform (Kalkhoven *et al.*, 1998). In these studies in Hep G2 cells I demonstrated that SRC-1a activated transcription by human ERα and ERβ1 to a greater extent than did SRC-1e. In previous publications SRC-1e was demonstrated to enhance hERα and hERβ activity to a greater extent than SRC-1a in Cos 7 cells (Kalkhoven *et al.*, 1998). The difference between the two sets of findings may be due to a number of cell-specific differences and the constructs used.

In co-transfection studies in Hep G2 cells the addition of SRC-1e stimulated gene transcription and appeared to oppose the dominant negative effect on ERα induced by the presence of ERβ1 or ERβ2. In co-transfection studies with ERβ1 and ERβ2, SRC-1e was observed to increased gene activation in the absence of ligand. This ligand-independent activation of the reporter may be due to alternative pathways being activated through the ERβ2.

7.3 Alternative Activation Pathways

The ERβ2 splice variant does not contain a fully functional ligand binding domain due to the lack of an AF-2 region, therefore the receptor cannot bind to ligands and become activated. It was previously thought that ERβ2 did not have a function in the regulation of oestrogen regulated genes, however in this thesis it has been

demonstrated that the expression of ERβ2 can enhance reporter gene activation in the absence of ligand when it is co-transfected with ERβ1. Therefore I proposed that it could be activated through a non-steroidal pathway such as via the MAP kinase signalling pathway which has been shown to activate ERα (Pettersson and Gustafsson, 2001) and mouse ERβ1 (Tremblay *et al.*, 1999a). This would be consistent with the presence of intact AF-1, DNA binding and dimerisation domains.

In this study I have demonstrated for the first time that phosphorylated MAP kinases (p44 and p42) are present in Hek 293 and Hep G2 cells even when they were cultured without serum or exogenous growth factors. I have also demonstrated that both cell lines express IGF receptor and Hek 293 cells express the EGF receptor (mRNA and protein) and therefore activation of the MAP kinase signalling cascade following the addition of growth factors is possible. In Hek 293 cells transfections using the FP-tagged ER have shown that ERα, ERβ1 and ERβ2 all redistribute from a diffuse distribution to a focal pattern in the presence of EGF. Functional studies have been performed and have demonstrated that the addition of EGF to Hek 293 cells transiently transfected with ERα, ERβ1 or ERβ2 and an ERE-Luc reporter construct resulted in increased gene activation. This result indicates that ERs, including ERβ2, are activated by phosphorylation from the MAP kinase pathway. This alternative pathway of ERβ2 activation is most likely most due to the phosphorylation of serine residues in the AF-1 domain. It has previously been demonstrated that hERα (Kato *et al.*, 1995) and mouse ERβ1 (Tremblay *et al.*, 1999b) are phosphorylated via the MAP kinase pathway at serine residues in the A/B domain, specifically Ser118 in ERα (Kato *et al.*, 1995) and Ser87 and Ser106 in ERβ (Rochette-Egly, 2003). These two serine residues in ERβ1 have been proposed to be phosphorylated by MAP kinases (Rochette-Egly, 2003) and peptide sequence alignment shows that human ERβ2 contains identical residues to human ERβ1 within the A/B domain.

7.5 Future work

Further studies on cell-specific expression of ERβ2 in the primate will require development of new antibody reagents. RT-PCR analysis has detected mRNA for ERβ2 within cDNA pools taken from the same range of primate tissues as those examined from human, suggesting that the primate ERβ2 protein may be expressed

in a similar pattern to the human. In human tissues it would be very interesting to perform dual or triple immunohistochemical staining for ERα, ERβ1 and ERβ2 to determine their differential expression in tissues and to show which cells express both receptors. However this has not been possible to date due to poor specificity of secondary antibodies.

In the transient transfection studies only 30% of the cells were transfected, therefore in the co-transfection studies it was difficult to achieve transfection of both receptors in the same cell. This low level of transfection efficiency was observed using FP-tagged receptors where the ER proteins could be visualised and from the immunocytochemical staining of transfected cells. This may have had an impact on the functional studies where variation between individual experiments was observed, most likely due to the inefficiency of dual transfections. Therefore I attempted to produce a stable Hek 293 cell line expressing either ERβ1 or ERβ2 tagged to the green fluorescent protein, to obtain a uniform level of expression of the ER. However after cloning the vectors, isolation of a stable line proved extremely time consuming and I decided to concentrate on the transient transfections instead. However, within our laboratory adenoviral constructs have been prepared using the FP-ERβ1 and FP-ERβ2 and as these result in more efficient transfection of cells future work using these adenoviral constructs in co-transfection studies will be undertaken to extend the current findings.

Since cloning the ERβ1 and ERβ2 constructs with the fluorescent tags, I think it would be extremely interesting to be able to observe the co-localisation of these receptors with the co-activator SRC-1. Stenoien *et al* have previously demonstrated that SRC tagged with the green fluorescent protein co-localised to the same nuclear matrix bound foci as ERα tagged with the yellow fluorescent protein, in response to E_2 (Stenoien *et al.*, 2000). Similar experiments have not been performed using ERβ1 or co-transfections with ERα and ERβ. Therefore future studies with the tagged SRC-1 and ERα or ERβ would be valuable in assessing the ability of ERβ homodimers or ERα/ERβ heterodimers to recruit SRC-1.

Preliminary data (Moore *et al.*, 1998; Scobie *et al.*, 2002) suggests that human tissues also express other ERβ variants (ERβ3, ERβ4 and ERβ5) different to the ERβ2 splice variant studied in this thesis. For example, in tissues such as the testes, ERβ4, which lacks a DNA binding domain, is abundant and therefore may

alter oestrogen regulation. These ERβ variants may therefore add to the complexity of the oestrogen and growth factor responsiveness of reproductive tissues. Additional studies are required to determine if co-expression of ERβ variants can alter ligand-dependent gene activation via full length ERα or ERβ1 and to determine whether they have significant physiological effects.

Future work into the activation of a non-steroidal pathway is required. I have only looked at one of the phosphorylation cascades and more work is required using techniques such as immunoprecipitations with specific antibodies to determine the phosphorylation status of the ERβ1 and ERβ2 in the presence and absence of EGF. Further work is also required to determine the precise pathway of activation of the ERβ2 and whether this activation pathway plays a large or a basal role in the transcription of genes. It would be interesting to block the MAP kinase pathway to determine whether other pathways are involved in ER activation. There are also other interacting proteins such as co-activators that are recruited upon the phosphorylation of the oestrogen receptor which need to be analysed.

I have shown that the EGF receptor is present in the testis within the membrane of germ cells in humans and primates, therefore demonstrating that the receptor is present in the same cell type as ERβ1 and ERβ2. It is necessary to now perform dual immunohistochemical studies on a wider variety of tissues to determine the exact localisation of these receptors and to establish whether this alternative phosphorylation pathway is feasible.

7.5 Conclusions

In summary the results obtained demonstrate that ERβ1 and ERβ2 are expressed in a variety of cells within reproductive tissues of females and males, in human and in two primates. Human ERα and human, macaque and marmoset ERβ1 are activated by a variety of oestrogenic ligands. The ERβ2 variant cannot be activated by binding of oestrogens due to the lack of an AF-2 region, but appears to be a target for phosphorylation via growth factor stimulated pathways. The ability of the ERβ2 "receptor" to form heterodimers with ERα and ERβ1 and thereby altering target cell responsiveness to oestrogens may have an impact on cell function. Further studies are now required to elucidate the relative contributions of ERα, ERβ1 and ERβ2 variants in the response of tissues to oestrogens and growth factors.

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Appendix to Materials and Methods

Company Addresses:

Active Motif:	Rixensart, Belgium
Adobe Systems:	Mountainview, CA
Advanced Biotechnologies (Abgene):	Surrey, UK
Ambion:	Abingdon, UK
American Type Culture Collection:	VA, USA
Amersham Life Sciences:	Buckinghamshire, UK
Berthold Technologies:	Hertfordshire, UK
BD Biosciences:	Oxford, UK
Bio-Rad Laboratories:	Hemel Hempstead, UK
Calbiochem:	Nottingham, UK
Cambridge Biosciences:	Cambridgeshire, UK
Cellstar ® Greiner bio-one:	Gloucestershire, UK
Clontech:	Hants, UK
Costar:	The Netherlands
DAKO Corp:	Cambridge, UK
Diagnostics Scotland:	Carluke, Scotland, UK
Eastman Kodak Co:	Rochester, NY, USA
European Collection of Cell Culture (ECACC):	Wiltshire, UK
Gene Therapy Systems (Cambridge Biosciences):	Cambridgeshire, UK
Genosys	Cambridgeshire, UK
Gibco:	Paisley, UK
GRI Syngene:	Essex, UK
Hoefer Scientific Instruments:	USA
Invitrogen:	The Netherlands
Kendro Laboratory:	Hertfordshire, UK
Leica:	Milton Keynes, UK
Microzone Ltd:	Lewes, UK

Millipore:	Watford, UK
MJ Reasearch:	MA, USA
MWG Biotech:	Wolverhampton Mill South, UK
Nalgene Nunc International:	Hereford, UK
New England Biosciences (NEB):	Hertfordshire, UK
Novel Experimental Technology:	UK
Olympus Optical Co:	London, UK
Pharmacia Biotech Qenequant:	Hertfordshire, UK
Photosol Ltd:	Essex, UK
Plastik® Cultureware, MatTek Coporation:	MA, USA
Promega:	Southampton, UK
Qiagen:	West Sussex, UK
Roche:	East Sussex, UK
Sigma:	Poole, Dorset, UK
Stratagene:	Amsterdam, The Netherlands
Tefal:	Nottingham, UK
Tocris Cookson Ltd:	Bristol, UK
Vector SP-2001:	Peterborough, UK
VH Bio Ltd:	Newcastle Upon Tyne, UK
Zeiss:	Hertfordshire, UK